

ISOLATION AND CHARACTERISATION
OF HISTONE TRANSACETYLASES

by

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ABBREVIATIONS

AcCoA	Acetyl Coenzyme A
cpm	Counts per minute
DEAE-	Diethylamino ethyl-
dpm	Disintegrations per minute
EDTA	Ethylene diamine tetracetic acid
MKT	3.5 mM MgCl_2 - 0.25 M KCl - 0.14 M Tris-HCl (pH 7.9)
OD_{230}	Optical density at 230 nm
PITC	Phenylisothiocyanate
PTH-	Phenylthiohydantoin
TEM	0.03 M Tris-HCl (pH 7.9) - 1 mM EDTA - 5 mM β -Mercaptoethanol
TKM	0.05 M Tris-HCl (pH 7.9) - 0.025 M KCl - 1.5 mM MgCl_2
Tris	Tris (hydroxymethyl)-aminomethane
μCi	Microcurie ($= 2.22 \times 10^6$ dpm)

Histone nomenclature

The histone nomenclature used throughout is that adopted at the Ciba Symposium during 1974 (Bradbury, 1975).

SUMMARY

Acetylation, one of the post-synthetic modifications of histones, weakens histone-DNA interactions and may play a regulatory role in gene control of eukaryotes. The literature available on histone acetylation as well as other post-synthetic modifications of histone has been reviewed.

Histone acetylation is catalysed by an enzyme(s) which transfers acetyl groups from a donor molecule to histones. A crude histone transacetylase preparation was isolated from nuclei and the optimal conditions for the acetylation of histones were determined. This enzyme(s) was shown to be specific for histones with protamine displaced histone complex being the best substrate.

Using this histone transacetylase preparation ^3H -acetyl total histone was prepared in sufficient yield and with a high enough specific activity to enable sequential Edman degradation of the histone sub-fractions isolated from the total histone complex to be undertaken.

Histones H3 and H4 were isolated from the acetylated total histone as they exhibited the highest degree of acetylation. Histone H4 peptides were generated by chymotryptic and tryptic digestion as the intact histone H4 polypeptide chain is blocked at its N-terminus.

The Edman degradations of histone H3 and H4 showed that the acetylation sites that have been determined under in vitro conditions are the same as those undergoing acetylation in vivo. All of the acetylation was found in the N-terminal region of histones H3 and H4 with histone H4 showing a gradient of decreasing acetylation from the N- to the C-terminus, in contrast to histone H3 where the first two possible acetylation sites are acetylated to a minor degree only. The major sites of acetylation in histones H3 and H4 were found to have the sequence

Acetyl
 -Gly - Lys - X - (X = Gly or Ala). The biological significance of these results has been discussed

"..... as for knowledge, it will pass away. For our knowledge is imperfect; but when the perfect comes, the imperfect will pass away..... For now we see in a mirror dimly, but then face to face. Now I know in part; then I shall understand fully."

1 Corinthians 13

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PART 1

INTRODUCTION

1.1 INTRODUCTION

Histone acetylation has been extensively studied (see 1.2.7) since the finding that acetylation of histones decreased their inhibitory effect on RNA synthesis (Allfrey et al., 1964).

The sites of in vivo acetylation of histones have been reported (see Table 1.2.7) and some of the enzymes responsible for this acetylation have been studied and are discussed in detail in the review which appears below (1.2.7). Dixon has studied the known sites of acetylation and concludes that there are two main amino acid environments for acetylation of lysine residues in histones to occur; either a small neutral amino acid (usually glycine but can also be alanine, serine or threonine) occurs on both sides of the acetyllysine or else the acetyllysine occurs as a member of a Lys-Lys, Lys-Arg or Arg-Lys pair. Dixon suggested that these two classes may reflect different specificities of at least two distinct histone transacetylases (Dixon et al., 1975a). DeLange has also studied the sites of acetylation of histones H3 and H4 and found that the two major sites of acetylation in histone H3 and the major site in histone H4 have the sequence -Acetyl-Lys-X-Y-Arg-Lys-. These authors (DeLange et al., 1972) have suggested that the same histone transacetylase is responsible for the acetylation at these three sites. Dixon has however stated that whether a given lysyl residue is acetylated probably depends to some degree also on certain aspects of the secondary structure of the histone as well as the amino acid sequence at the acetylation site (Dixon et al., 1975b).

Various authors have mentioned that the specificity of the histone transacetylases may depend on a certain conformation of histones resulting from their interaction either with DNA (Gallwitz, 1970b,

Gallwitz and Sures, 1972, 1973) or else with other histones in specific histone aggregates (Candido, 1975). Since attempts to detect acetylation of histones after in vitro recombination with DNA have been reported to be unsuccessful (Gallwitz and Sures, 1972), it was decided to use as a substrate for the histone transacetylase a histone complex obtained by the protamine displacement method of van der Westhuyzen and von Holt (1971), which has been reported (Kornberg and Thomas, 1974) to contain specific histone aggregates.

Multiple functions for histone acetylation have been put forward (see 1.2.7) some of which could be expected to occur in the cytoplasm, but mostly to be located in the nucleus. It has been suggested (Smith, 1975a) that a "battery" of histone modifying enzymes e.g. histone transacetylases, is required in the cell and indeed multiple histone transacetylases have been reported in the nucleus and cytoplasm (Harvey and Libby, 1976; Gallwitz, 1971; Gallwitz and Sures, 1972).

A knowledge of the specificity and mode of action of histone transacetylases may assist in the understanding of the way newly synthesised histones and DNA are assembled in chromatin (Candido, 1975). Dixon's group has also said that further studies of the substrate specificity of the histone transacetylases may help to yield clues to the structural assembly of chromosomes and the mechanism of chromatin coiling and uncoiling (Louie et al., 1973). It is clearly a necessity to study the histone transacetylase mechanism and specificity further before we can answer the question recently asked by Smith (1975a) of "How do the specific enzymes that catalyse the various known (histone) modifications recognise the specific sites?" The same author also stated in his concluding remarks at the end of a recent symposium that acetyltransferases (as well as other chromatin proteins) should be more precisely characterised and defined so that, (a) their properties might be correlated with the properties of the non-histone protein fraction and (b) that the assembly and functional role of the histone transacetylases separately and as part of chromatin might be better known (Smith, 1975b).

The present work reports the sites of enzymatic acetylation of the histone H3 and H4 molecules catalysed under in vitro conditions which had not been previously reported despite the fact that various histone transacetylases have been prepared (see 1.2.7) and characterised with respect to their affinity to the various histone subfractions.

1.2 A REVIEW OF POST SYNTHETIC HISTONE MODIFICATIONS

1.2.1 Introduction

Four of the five histone types occurring in eukaryotes have been sequenced (Sautierre, 1973; Iwai et al., 1970; DeLange et al., 1969a, 1972). Histone H4 has the lowest mutation rate of any protein studied, only 2 of the 102 amino acid residues differing between pea and calf histone H4 and these are conservative substitutions with valine at residue 60 in calf being replaced by isoleucine and lysine 77 being replaced by arginine in pea (DeLange et al., 1969a,b). Histone H3 has also been highly conserved throughout evolution with only 4 amino acid substitutions between pea and calf histones H3 (DeLange et al., 1972; Patthy et al., 1973). Sequence studies also indicate that the basic residues are irregularly clustered, histones H3, H2B and H4 have very basic amino terminal ends, while H2A has a basic region in both the amino and carboxyl terminal regions. Histone H1 in contrast has a basic carboxyl terminal region (Elgin and Weintraub, 1975). In each case clustering of the hydrophobic amino acids occurs in the central region of the histone molecule. It has been suggested (DeLange et al., 1969a) that the basic regions would be involved in histone-DNA interaction with the remainder of the molecule being involved in interactions with other histones or non-histone proteins.

Although the amino acid sequences of histones, particularly histone H3 and H4, have been highly conserved during evolution, a wide range of post-synthetic modifications are known. Presently known modifications are methylation, phosphorylation, ADP-ribosylation and acetylation most of which occur in the basic N-terminal region (see below). Each of these modifications is briefly discussed below, as well as histone proteolysis and sulphhydryl-disulphite interconversion of SH-containing histones. A more detailed discussion of histone acetylation is presented since it was the subject of the present study.

1.2.2 Histone methylation

Since histone methylation has recently been extensively reviewed (Paik and Kim, 1975; Cantoni, 1975; Byvoet and Baxter, 1975), as well as having been discussed in recent reviews of histones or chromosomal

proteins (DeLange and Smith, 1971; DeLange and Smith, 1975; Stellwagen and Cole, 1969; Hnilica, 1972; Allfrey, 1971; Dixon et al., 1975), only a brief summary of the subject will be presented here which will concentrate on the chemical characterisation of the phenomenon as well as postulated biological functions for histone methylation.

Altogether seven methylated amino acids have been isolated from histones from various organisms : mono-, dimethyllysine (Paik and Kim, 1967) or trimethyllysine (Burdon and Garven, 1971), N^G -monomethylarginine (Paik and Kim, 1970; Byvoet, 1971), $N^G N^G$ - or $N^G N^{G'}$ -dimethylarginine (Kakimoto and Akazawa, 1970) as well as 3-N-methylhistidine (Byvoet, 1971). The methyl groups are incorporated into the polypeptide chain after it has been synthesised (Sekeris et al., 1967; Kim and Paik, 1965). Some of the enzymes responsible for this methylation have been isolated (Paik and Kim, 1975; Gallwitz, 1971) and their substrate specificity as well as intracellular locations are shown in Table 1.2.2.1.

TABLE 1.2.2.1

METHYLATING ENZYMES

Enzyme	Amino acid side chain methylated	Location	Reference
Protein methylase I	Arg	cytosol	Paik & Kim (1975)
Protein methylase II	-COOH of Glu or Asp	cytosol	
Protein methylase III	-Lysine	nuclear	
Histone methyl-transferase I _N	Histone H3 (amino acid residues not reported)	nuclear	Gallwitz (1971)
Histone methyl-transferase II _N	Arg in H4	nuclear	

All of these enzymes use S-adenosyl-methionine as methyl donor. Paik and Kim (1975) have suggested that more than one enzyme may be required to form di- or trimethyllysine. Cantoni has suggested that "the presence of a variety of methylated proteins and the specificity of the methylation patterns (found in various systems) suggest a greater complexity in the specificity and distribution of protein methylases than has been discovered so far" (Cantoni, 1975). The existence of a histone demethylase has been claimed (Paik and Kim, 1973) but controversy still exists as to whether histone methyl groups are subject to turnover with respect to the polypeptide chains or not (Honda et al., 1975a).

Histones H3 and H4 are the histones which seem to be most susceptible to methylation (Hnilica, 1972; Honda et al., 1975b) although H1 methylation has been observed (Marsh and Fitzgerald, 1973), and H2B methylation of arginine residues has also been reported (Byvoet and Baxter, 1975). The major sites of methylation of histones H3 and H4 have been determined and are shown in Table 1.2.2.2. It is interesting that the major sites of methylation in H3 and H4 are 3-4 residues (which is 1 turn of the α -helix) from a lysine residue which can undergo acetylation, and in addition Ser 10 and Ser 28 in H3 can be phosphorylated as well. This could indicate a possible link between methylation and acetylation or phosphorylation. The amount of methylated histone varies in individual tissues and perhaps with the functional activity of the cells (Paik and Kim, 1971; Hnilica, 1972). The major methylation found is methylation of lysine residues with methylation of arginine, histidine and carboxyl groups of aspartic and glutamic acid being very minor when compared with lysine methylation.

It has been suggested that "the pK_b value of the ϵ -NH₂ group of lysine residues may approximate 3.37. When the first and second methyl groups are introduced into lysine, the pK_b value of the mono- and dimethyl lysine groups may be 3.22 and 4.20 respectively. Substitution with a third methyl group results in the formation of a strongly basic quarternary ammonium ion. Therefore the basicity scale will be di-<non-<mono-<trimethyllysine in increasing order" (Kim, 1971). From the relative basicities it can be expected that dimethyllysine is less strongly ionically bound (presumably to DNA PO₄ groups) than non-methylated lysine and that mono- or trimethylation causes a stronger

ionic interaction. Alternatively the non-polar methyl group increases the hydrophobicity of proteins and could change hydrophobic interactions of the histone. Cantoni has discussed an interesting situation which occurs in cytochrome C where amino acid residues 76-87 which contain 2 lysine methylation sites in higher plants (Boulter et al., 1970) show no mutation at this site when compared with vertebrates which are not methylated here. From knowledge about the evolutionary relationships of cytochrome C of fungi, plants and vertebrates, it can be surmised that the methylated cytochromes branched from the phylogenetic tree 1-2 billion years ago (Dayhoff et al., 1972; Cantoni, 1975). Over this long period of evolution the region containing the methylatable residues has not mutated at all. The mutation frequency increases with distance from this sequence. This indicates a vital functional importance for the site 76-87 for cytochrome C (Cantoni, 1975). Similarly H3 and H4 which are even more highly conserved are also subject to methylation as well as other side chain modifications which presumably have functional or structural importance. The evolutionary stability of these histones are also of course determined by their requirements for interaction with (a) DNA phosphate groups, (b) other histones, and (c) non-histone proteins.

The peak of arginine methylation in histone H3 coincides with that of DNA synthesis in the Hela S-3 cell cycle, suggesting that the role of arginine methylation might be different from the role of lysine methylation, whose peak follows that of DNA synthesis by 3-6 hours (Paik and Kim, 1975; Borun et al., 1972). The role of arginine methylation is however unknown at this stage (Paik and Kim, 1975). During pancreatic regeneration histone H1 methylation occurs before DNA synthesis as well which implies that H1 methylation has a different function to the methylation of other histone subfractions (Marsh and Fitzgerald, 1973).

Histone methylation is a late event in the cell cycle (Honda et al., 1975a; Paik and Kim, 1975; Tidwell et al., 1968) occurring at a time when the rates of histone synthesis and DNA synthesis have begun to decline. Histone methylation is also postulated to correlate with structural and functional changes known to occur in the nucleus prior to or during mitosis, involving a condensation of the chromatin (Tidwell et al., 1968; Paik and Kim, 1975). Honda, Candido and Dixon have studied histone H4 metabolism in developing trout testes (Honda et al.,

1975a) and conclude that the order of events is : H4 synthesis followed by acetylation, deacetylation, methylation and phosphorylation. These authors suggest that "it is possible that histone methylation is a necessary prelude to phosphorylation and subsequent chromatin condensation. This methylation may involve changes in charge, conformation or hydrophobicity of the histone, allowing interactions with a histone phosphokinase or other molecules" (Honda et al., 1975a).

Paik and Kim (1975) have also suggested that "methylation of histone lysine residues is necessary for a proper conformation of chromatin after newly synthesized histone-, DNA- and non-histone proteins are assembled." Byvoet and Baxter (1975) have hypothesised that methylation would serve to more firmly lock the histones in place by increasing their binding to other macromolecules, to form a stable chromatin complex such as the 11S particles of Noll (1974) or the V-bodies proposed by Olins and Olins (1974). Byvoet and Baxter (1975) also suggest that the DNA, histones and methyltransferase must be in very close proximity in pre-mitotic chromatin because very small changes in DNA structure caused by a small polylysine (9 lysines) binding to DNA cause a 100% stimulation of histone methylation. Another possible function of histone methylation is that it could cause modification of histone proteolysis (see below). In summary it may be concluded that the function of histone methylation is at this stage still largely speculative but is probably involved in conformational changes of the chromatin prior to or during mitosis.

1.2.3 Histone phosphorylation

Histone phosphorylation like histone methylation has recently been reviewed (Langan and Hohmann, 1975; Ord and Stocken, 1975; Dixon et al., 1975) or else mentioned in recent reviews of histones (Allfrey, 1971; Hnilica, 1972; Elgin and Weintraub, 1975; DeLange and Smith, 1975). Histone phosphorylation has also been discussed in a recent review of protein phosphorylation (Rubin and Rosen, 1975).

Histone phosphorylation takes place after synthesis of the polypeptide chain (Kleinsmith et al., 1966) and utilises ATP as the phosphate group donor with serine or threonine hydroxyl groups as the main phosphate acceptors via an o-ester linkage (Ord and Stocken, 1966), with phospho-

histidine (Smith et al., 1973) or phospholysine (Chen et al., 1974) also having been reported.

Hnilica (1972) has pointed out that in evaluating studies of histone phosphorylation care must be exercised in the evaluation of results because of possible contamination of the histones by highly phosphorylated contaminants. In cell cycle studies the methods used to synchronise the cells, the degree of synchrony obtained as well as the cell type can influence the results obtained (Rubin and Rosen, 1975). The chemical characterisation of histone phosphorylation will be concentrated upon here with only a brief treatment of the enzymology and histone subfraction susceptibility to phosphorylation being presented.

Histone phosphorylation is known to be a reversible process in contrast to methylation (Stevely and Stocken, 1966). Various histone phosphokinases which are responsible for the phosphorylation are known to consist of catalytic and regulatory subunits and to be either cyclic nucleotide monophosphate (especially cAMP)-dependent or-independent and to be localised in both the nucleus and cytoplasm (Hnilica, 1972; Langan, 1971; Chen and Walsh, 1971; Majumder and Turkington, 1971; Yamamura et al., 1970; Nesterova et al., 1975; Rubin and Rosen, 1975). Langan has also shown that in H1 histone one site (Ser 37) is phosphorylated by the cAMP-dependent protein kinase and another site (Ser 106) is modified by a cAMP independent kinase in resting tissue (Langan, 1971). A growth associated phosphokinase was shown to phosphorylate both serine and threonine residues in H1 (Langan and Hohmann, 1975). A histone phosphatase has also been isolated (Meisler and Langan, 1969).

A wide range of cell types and systems display histone phosphorylation with different patterns of histone phosphorylation for each subfraction, tissue or species (Allfrey, 1971; Hnilica, 1972). Histone H1 is the most phosphorylated subfraction in most systems but there are reports of all subfractions being phosphorylated. Histone phosphorylation is reported to be tissue and species specific with respect to the number of sites phosphorylated and the extent of phosphorylation with phosphorylation being greater generally in growing than in resting cells (Langan and Hohmann, 1975; Sung et al., 1971). Phosphorylation of H1 histone in contrast to the other subfractions is sensitive to radiation (Gurley and Walters, 1971) which suggests a different function for H1 phosphorylation

TABLE 1.2.3

PHOSPHORYLATION SITES IN HISTONES

<u>Group Ia</u>			<u>Group Ib</u>				
	PO ₄	(Ac)	Ref:	(Ac)	PO ₄	(Me)	Ref:
H2A	Ac-Ser-Gly-Arg-Gly-Lys-		a-d	H4	Ala-Lys-Arg-His-Arg-Lys-Val-		f-j
	1	5			16	18	20
	PO ₄	(Ac)			PO ₄		
H4	Ac-Ser-Gly-Arg-Gly-Lys-		a,b,e		Thr-Glu-His-Ala-Lys-Arg-		h,i
	1	5			75		
<u>Group IIa</u>			<u>Group IIb</u>				
	(Me) PO ₄			(Ac) PO ₄			
H3	-Arg-Lys-Ser-Thr-Gly-		c,j-n	H2B	-Ala-Lys-Ser-Ala-Pro		k,c
	10				6		
	(Me) PO ₄						
	-Arg-Lys-Ser-Ala-Pro-		c,j, l-n				
	28						
<u>Group III</u>			<u>Group III</u>				
	PO ₄				PO ₄		
H2A	-Thr-Arg-Ser-Ser-Arg-		o	H2B	-Lys-Gly-Ser-Tyr-Ser-Val-		o
	19				36		
	PO ₄				PO ₄		
H2B	-Lys-Gly-Ser-Lys		o	H2B	-Lys-Arg-Ser-Arg-		p
	14				32		
<u>Group IV</u>			<u>Ref:</u>				
	PO ₄						
H1	-Lys-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val-		b,q				
	38						
	PO ₄						
	-Gly-Ala-Ser-Gly-Ser-Phe-Lys-		q				
	106						
	PO ₄						
	-Ala-Ala-Lys-Lys-Ser-Pro-Lys-Lys-		c				
	157						
	PO ₄						
	-Ala-Ala- -Lys-Ser-Pro-Lys-Lys-		c				
	172						
	PO ₄						
	-Ala-Ala- -Lys-Ser-Pro-Lys-Lys-		c				
	196						

References to Table 1.2.3

a	Phillips, 1968	j	Honda et al., 1975b
b	Sung and Dixon, 1970	k	Candido and Dixon, 1972b
c	Dixon et al., 1975	l	DeLange et al., 1972
d	Candido and Dixon, 1972a	m	Brandt et al., 1974a
e	Dixon et al., 1969	n	Marzluff and McCarty, 1972
f	Candido and Dixon, 1971	o	Shlyapnikov et al., 1975
g	DeLange et al., 1969	p	Hashimoto et al., 1975
h	Smith et al., 1973	q	Langan et al., 1971
i	DeLange and Smith, 1975		

when compared with phosphorylation of the other fractions (Allfrey, 1971; Hnilica, 1972).

Many of the sites of phosphorylation are known and are shown in Table 1.2.3 which is a modified and updated version of the table presented by Dixon (Dixon et al., 1975c). Other substitutions (e.g. acetyl and methyl) which are known are shown in parentheses. The sites fall into several distinct groups as has been outlined by Dixon. Group Ia consists of the N-terminal sequences of H2A and H4 which are identical. Group Ib contains the histidine residues of H4 which are postulated to be able to undergo phosphorylation; these are the only additional sites in H4 which have been reported to be phosphorylated. Group IIa contains a Lys-Ser sequence where the Lys can undergo methylation, suggesting that the first modification may alter the specificity of the enzyme catalysing the second modification (Dixon et al., 1975d). Group III shows recently discovered sites in H2A and H2B, in every case the phosphorylated serine is separated by one amino acid from either arginine or lysine (Williams, 1976). Group IV contains the known phosphorylated sites of histone H1. All of the H1 sites with the exception of Ser 38 are in the COOH terminal region of H1 in contrast to all the other histones whose modifications occur in the N-terminal region (Dixon et al., 1975c). The three sites nearest the C-terminus all have the sequence Lys-Ser-Pro-Lys-Lys. From the differing sequences at the sites of phosphorylation it is reasonable to assume that a number of phosphokinases of different specificity are involved in histone phosphorylation.

A number of possible functions for histone phosphorylation have been

suggested and some of these are mentioned below. A more detailed treatment is presented in recent reviews (Rubin and Rosen, 1975; Langan and Hohman, 1975; Elgin and Weintraub, 1975; Dixon et al., 1975; DeLange and Smith, 1975). Newly synthesised histones undergo very rapid phosphorylation which is suggested to be necessary in conjunction with other modifications, especially acetylation, for the deposition and correct binding of new histones to DNA (Ruiz-Carrillo et al., 1975; Jackson et al., 1976; Louie et al., 1973). Histone H5 phosphorylation may be involved in the effect of H5 in immature cells on condensation and template restriction of chromatin which occurs in the terminal stages of differentiation of the avian erythroid cells (Tobin and Seligy, 1975). Gurley et al. (1975) studied histone phosphorylation in the Chinese hamster cell cycle and concluded that H1 phosphorylation which occurs during G₁ phase is involved in chromatin structural changes necessary for cell proliferation; while phosphorylation during S phase involved in DNA replication and H1 phosphorylation occurring in mitosis concurrently with H3 phosphorylation is involved in chromosome condensation. Further support for H1 phosphorylation having a multiple function is that the sites of phosphorylation found in S and M phases are reported to be different (Langan and Hohmann, 1975).

Histone H1 phosphorylation has been interpreted as being important in the control of chromosome replication rather than modification of transcription (Balhorn et al., 1972). H1 phosphorylation has been reported to increase before the synthesis of both, RNA (Allfrey, 1971) and DNA (Stevely and Stocken, 1968), occurs which may possibly be due to increased levels of phosphokinase activity (Pawse et al., 1971). A correlation between histone phosphorylation and chromosome condensation has been made in a wide variety of cells (Rubin and Rosen, 1975). It has been proposed by Bradbury's group that cell division in the slime mold Physarum polycephalum is triggered by histone H1 phosphorylation (Bradbury et al., 1974), and that a histone phosphokinase is the actual "mitotic trigger" (Inglis et al., 1976). Histone H1 phosphorylation as a requirement for the G₂ → M transition has been established by Lake (1973). Marks et al. (1973) have concluded that H1 phosphorylation is related to the separation and condensation of the chromatids before and during mitosis. Phosphorylation of H1 may be expected and may even be necessary when structural alterations in the nucleus are required (e.g. during replication or mitosis) but at this stage it is not justified

in thinking that this is the sole cause of the event (Ord and Stocken, 1975). Histone phosphorylation may also be necessary for the formation of ADP-ribose derivatives, e.g. a phosphorylated serine residue could be required for the formation of phosphoseryl-poly(ADP ribose) which has been proposed by Smith and Stocken (1975).

In summary histone phosphorylation can be implicated in many intracellular functions with histone H1 being the most conspicuous substrate for phosphorylation-dephosphorylation. Nevertheless, however important phosphorylation is by itself, it seems to be closely correlated with other dynamic post-synthetic modifications (especially acetylation) which will be further discussed below.

1.2.4 Adenosine diphosphoribosylation (ADP-ribosylation) of histones

Nuclei are capable of catalysing the polymerisation of the ADP-ribose moiety of NAD into a homopolymer, with the simultaneous release of nicotinamide (Chambon et al., 1966). The ADP-ribose units are thought to be linked through the ribose moieties, position 1' on the NMN ribose and 2' on the adenine ribose being linked by a glycosidic bond (Chambon et al., 1966; Hasegawa et al., 1967; Reeder et al., 1967). The enzyme system poly(ADP-ribose) polymerase has been partially purified from chromatin (Yoshihara, 1972) and the presence of at least several enzymes dealing with poly(ADP-ribose) metabolism has been indicated (Dietrich et al., 1973).

The length of the polymer varies from monomer to 10-30 ADP-ribose units (Sigimura et al., 1971). Linkage of the polymer to protein is covalent, but the nature of the linkage is not known, but neutral hydroxylamine-sensitive and -resistant linkages have been demonstrated, which suggest at least 2 types of linkage. Ueda et al. (1972) have suggested that the hydroxylamine labile linkage is an ester between the C₂ hydroxyl group of the ribose moiety and a carboxyl group in the protein. A second linkage which is hydroxylamine stable has been shown to be via serine phosphate by Smith and Stocken (1975), who also suggested that the hydroxylamine labile linkage might be via a phosphohistidine or phospholysine residue which is extremely acid labile which could explain the lability of this linkage.

Histone H1 has been found to be the fraction which mainly undergoes this modification (Smith and Stocken, 1973; Gilmour, 1974). The function of ADP-ribosylation of histones is not known but Yoshihara and Koide (1973) have shown that ADP-ribosylation of nuclear proteins results in a liberation of the DNA polymerase from chromatin in vitro and Ord and Stocken (1974, 1975) have suggested that ADP-ribosylation of H1 histone may either be responsible for the termination of DNA synthesis, or that the ADP-ribose by introducing a cluster of negative charges into the highly basic C-terminal of the molecule, might weaken histone H1-DNA interactions in transcriptionally active regions.

1.2.5 Thiolation

In higher eukaryotes, H3 is the only histone which contains cysteine (DeLange and Smith, 1975). Histone H4 from echinoderms has been shown to also contain cysteine (Subirana, 1971; Strickland et al., 1974). Early studies showed that the histone H3 found in plants, invertebrates and vertebrates up to and including rodents has only one cysteine residue, while the H3 histone of more advanced species possessed 2 cysteines per H3 molecule (Panyim et al., 1971). Recently however two forms of H3 in calf have been found with about 20% of the molecules having only one cysteine residue (Patthy and Smith, 1975), with serine in place of cysteine at position 96. Garrard (1976) has also shown that rat H3 contains at least 1% of a component which contains at least 2 cysteine residues.

Palau and Padros (1972) found that the 2 cysteic residues in calf thymus H3 were in different environments, one was located on the surface and was very reactive, and the other is buried within the tertiary structure of H3. No similar information is known about the SH-groups in the H4 echinoderm histones.

Sadgopal and Bonner have claimed that histone H3 occurs mainly in the reduced monomeric form in interphase chromosomes, while in metaphase chromosomes, these sulphydryl groups are oxidised to the disulphide form and histone H3 is either polymerised or complexed with acid-soluble, non-histone proteins through interpolypeptide disulphide bonds (Sadgopal and Bonner, 1970). These disulphide bonds could serve to stabilise the supercoiled structure or else to be involved in chromatin

condensation (Sadgopal and Bonner, 1970). However the fact that histone H3 may contain 1 or 2 sulfhydryl groups points at the non-importance of at least one cysteine. In developing sea urchin embryos the thiol content increased in the early cleavage stages (Ord and Stocken, 1968, 1969). It appears that changes in the thiol-disulphide content correspond with changes in the structural state of the chromatin during replication or mitosis (Hnilica, 1972; Ord and Stocken, 1975).

1.2.6 Proteolysis

Another post-synthetic modification which histones can undergo is proteolysis. The origin of histone proteolytic activity is controversial. Furlan and Jericijo (1967a,b) reported two histone proteases with pH optima of 4.4 and 7.8. They suggested that the neutral protease is located in the nuclei, whereas the acid protease was due to cellular contamination of the nuclei. Other reports have shown the presence of a neutral protease in nuclei or chromatin (Kurechi and Toczko, 1972; Kurecki et al., 1971; Garrels et al., 1972; Bartley and Chalkley, 1970). A cytoplasmic origin for the neutral protease has however been indicated by the findings of other workers (Destree et al., 1975; Stellwagen et al., 1968; Heinrich et al., 1976). Destree and coworkers (Destree et al., 1975) have concluded that the neutral proteolytic activity which causes degradation of histones in isolated chromatin is due to contamination with neutral protease(s) from cytoplasmic organelles. Heinrich et al. (1976) have found that the only organelles which contained histone-degrading enzymes are lysosomes and mitochondria with the enzyme activities having pH optima of 4-5 and 7.5-8.0 respectively. They conclude that the neutral nuclear protease is due to contamination with the mitochondrial enzyme.

Due to the doubt concerning the localisation and purification of the nuclear enzyme(s), studies of histone subfraction susceptibility to the protease(s) as well as the biological significance of these enzyme(s) have to be interpreted with caution.

Limited proteolysis of H1 histone by the mitochondrial membrane-bound protease has been reported by Haas et al. (1976) with a limit digested product with a molecular weight of about 13 000 having been reported.

Further work is however required before it can be stated whether the mitochondrial enzyme has any physiological role in the degradation of chromosomal proteins. The nuclear neutral protease is reported to only degrade H1 and H3 when they are complexed in chromatin, but to degrade all subfractions when they are dissociated from DNA (Kurecki et al., 1971; Bartley and Chalkley, 1970). Contrary to these results Garrels et al. (1972) reported that all chromatin-bound histone subfractions were degraded. Proteolytic degradation of histones H3 and H4 has also been reported by the chromatin bound protease by Brandt et al., (1974b).

It has been suggested that modification (e.g. by phosphorylation or acetylation) of DNA-bound histone could loosen the histone binding and make the partially dissociated histone more susceptible to proteolysis (Rubin and Rosen, 1975; Dixon et al., 1975; Garrels et al., 1972). Brandt et al. (1974b) have found that H3 and H4 are degraded at the C-terminal side of lysine 23 or 16 respectively. Both of these lysines can be acetylated (Table 1.2.7) which could alter the susceptibility of these bonds to proteolytic degradation (Brandt et al., 1974b) as could phosphorylation or methylation of nearby serine or lysine residues (Table 1.2.3 or Table 1.2.2.2). Seely and Benoiton (1970) have however found that methylation imparts little or no protective effect from proteolytic enzymes such as trypsin to polypeptides or histone. A further interesting feature is that all of the sites which are reported to be able to be acetylated in H3 and H4 (see Table 1.2.7) are on the N-terminal side of these cleavage points and would be removed by proteolysis. Whether proteolysis of histones is purely a random process or whether it is involved in specific derepression of previously repressed genes will have to await further investigations.

1.2.7 Histone acetylation

Since the original finding that acetylation of histones decreased their inhibitory effect on RNA synthesis (Allfrey et al., 1964) it has been shown that histone acetylation takes place after synthesis of the polypeptide chain (Allfrey et al., 1964; Pogo et al., 1966; Gallwitz and Sekeris, 1969) and can be divided into metabolically stable and labile classes (Wilhelm and McCarty, 1970; Jackson et al., 1975; Marzluff and McCarty, 1970). The sites of acetylation (see Table 1.2.7) and

acetyl amino acid derivatives have been identified (Gershey et al., 1968; Vidali et al., 1968; DeLange et al., 1969) and some of the enzymes responsible for the acetylation and deacetylation processes have been isolated (see below).

Histone acetylation has been studied during the cell cycle (Shepherd et al., 1971, 1972a, 1972b), and the effect on histone acetylation in different systems of various stimulatory agents such as hormones (Libby, 1968, 1972, 1973; Ono et al., 1969; Burdon and Pearce, 1971; Pearson and Paik, 1972; Graaff and von Holt, 1973; Liew et al., 1973), liver regeneration after partial hepatectomy (Jones and Irvin, 1972; Oh and Conard, 1972a; Gilmour, 1974) and pancreatic regeneration (Marsh and Fitzgerald, 1973) as well as the effects of ageing (Oh and Conard, 1972a, 1972b; Ryan and Cristofalo, 1972), radiation (Shepherd et al., 1972b) and drugs (Takaku et al., 1969; Edwards and Allfrey, 1973; Brown and Liew, 1975) have been investigated.

Most of these studies indicated that the level of histone acetylation is linked to the level of general metabolic activity of the cell. An increase in histone acetylation was reported to occur before an increase in RNA synthesis (Pogo et al., 1966; Liew et al., 1973; Graaff and von Holt, 1973; Takaku et al., 1969) while Marsh and Fitzgerald (1973) found that an increase in histone acetylation correlated with DNA synthesis. These studies suggest multiple functions for histone acetylation in the cell.

As this thesis has concentrated on the chemistry and enzymology of histone acetylation, these aspects will be discussed below in some detail. A recent model for one of the functions of histone acetylation will also be discussed.

Histones H1, H2A and H4 have N- α -acetyl serine at the N-terminal end of the polypeptide chain (Rall and Cole, 1971; Sautiere, 1974; DeLange et al., 1969). Since these histones are always blocked at the N-terminus it is assumed that the acetyl group is not subject to metabolic turnover. It has been suggested that N-acetyl-seryl-tRNA participates in the initiation of synthesis of histones (Liew et al., 1970) analogously to the role played by N-formyl-methionyl-tRNA. In contrast it has been

shown that methionine initiates the in vitro synthesis of histone (Gilmour and Dixon, 1972; Gallwitz and Sures, 1974; Kecskes et al., 1976). Pestana and Pitot (1974) also suggested that an enzyme (which still has to be isolated) can acetylate nascent peptide chains and that acetyl serine is not required as an initiator. Another suggestion is that histones H1, H2A and H4 are initiated with methionine like other proteins and then an enzymatic mechanism involving a tRNA may modify the N-terminus by transferring acetyl-serine to the N-terminal amino acid (Allfrey, 1975). At this stage the mechanism of terminal N-acetylation of the histones is still unknown.

This thesis investigated the internal enzymatic acetylation of histone which occurs on the ϵ -NH₂ group of lysine residues with the formation of N- ϵ -acetyllysine (Vidali et al., 1968; DeLange et al., 1969) within the polypeptide chain. This modification is metabolically labile and the acetyl groups are subject to rapid removal from the polypeptide chain (Allfrey et al., 1964; Wilhelm and McCarty, 1970; Marzluff and McCarty, 1970; Johnson et al., 1973; Jackson et al., 1975). Histones can also be O-acetylated (Nohara et al., 1966, 1968; Gallwitz and Sekeris, 1969) presumably on serine or threonine residues. The site(s) of this modification and derivative(s) formed have not yet been identified. The acetyl group donor for histone acetylation is acetyl Coenzyme A (Gallwitz, 1968; Nohara et al., 1968; Gallwitz and Sekeris, 1969; Racey and Byvoet, 1971). Non-enzymatic acetylation occurs in the presence of acetyl-CoA at pH values above 9 with acetyllysine being formed (Gallwitz, 1968, 1970a; Gallwitz and Sekeris, 1969; Paik et al., 1970). Histone fractions H4 and H3 have been found to be the best acceptors of the enzymatically catalysed transfer of labile acetyl groups (Allfrey et al., 1964; Pogo et al., 1966; Nohara et al., 1966, 1968; Vidali et al., 1968; Wilhelm and McCarty, 1970; Horiuchi and Fujimoto, 1972; Johnson et al., 1973; Candido, 1975), a result which was confirmed in the present study (refer to part 3 below).

The known sites of acetylation in the various histone subfractions are shown in Table 1.2.7. (Nearby methylation and phosphorylation sites are shown in parentheses). Dixon has compared the sequences around all of the known acetylated lysyl residues and concludes that there are two main categories : "Type A is an acetyllysine with a small neutral amino acid on either side which is most often glycine but can be alanine, serine

or threonine; Type B has acetyllysine present as a member of a Lys-Arg, Arg-Lys or Lys-Lys pair." Dixon has suggested that these two classes may reflect differing specificities of at least two distinct histone transacetylases (Dixon et al., 1975a,b). In calf thymus the two major sites of N-ε-acetylation in histone H3 as well as the major acetylation site in H4 all have the sequence -Lys(Ac)-X-Y-Arg-Lys- and DeLange has suggested that the same histone transacetylase is responsible for the acetylation at these three sites (DeLange et al., 1972; Dixon et al., 1975b). Sequences alone are however unlikely to provide the only recognition signals for acetylation. Thus whether or not a given lysyl residue is acetylated probably depends to some degree also on certain aspects of the secondary structure of the histone as well as the amino acid sequence at the acetylation site (Dixon et al., 1975b).

The study of histone transacetylase enzymes was initiated by Nohara who isolated a crude extract from pigeon liver (Nohara et al., 1966, 1968) which was capable of incorporating both O- and N-acetyl groups into histones, with the arginine-rich histones being the fractions most susceptible to acetylation. Rat liver nuclei were shown (Gallwitz, 1968; Gallwitz and Sekeris, 1969) to contain an enzymatic activity which could transfer acetyl groups from acetyl-CoA to histones with H3 histone as the best substrate followed by H2A/H4 then H2B and H1. In vitro acetylation of all of the histone fractions was also observed by Bondy et al. (1970), who isolated the acetylation enzyme from nuclei of rat liver and brain. The enzyme was shown to be specific for histones and to have the same substrate specificity as the preparation of Gallwitz described above. Chromatin-bound histones were found to be less acetylated than free histone. Exposure of chromatin to solubilised histone transacetylase preparation increased its capacity to act as a template for RNA synthesis (Bondy et al., 1970) which supports the concept (Allfrey et al., 1964) that acetylation of histones within the chromatin complex may result in de-repression of the DNA genome. The acetylation rates of histones change during the cell cycle (Shepherd et al., 1971) but these changes were not regulated by the availability of histone transacetylase since histone transacetylase activity was found to remain constant in synchronised CHO cells (Noland et al., 1971). Purified histone transacetylases have been obtained from isolated nuclei of various rat tissues (Gallwitz, 1970a, 1971b; Gallwitz and Sures, 1972). Thymus contained three histone transacetylases, two of which (B₁ and B₂) preferred H4 as their substrate, although other

TABLE 1.2.7

ACETYLATION SITES IN HISTONES

<u>Histone</u> :		<u>Refs.</u>
<u>H1</u>	Ac-N-Ser-Glu-Ala-Pro-Ala- 5	(a)
<u>H2A</u>	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">(P) Ac-Ser-Gly-Arg-Gly-Lys-Thr-Gly-Gly-Lys-Ala-Arg-Ala-Lys-Ala-Lys-Thr-Arg-</div> <div style="text-align: center;">Ac 5</div> <div style="text-align: center;">10</div> <div style="text-align: center;">15</div> </div>	(b,d-f)
<u>H2B</u>	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> Ac (P) Pro-Glx-Pro-Ala-Lys-Ser-Ala-Pro-Lys-Lys-Gly-Ser-Lys-Lys-Ala-Val-Thr-Lys-Ser- </div> <div style="text-align: center;">5</div> <div style="text-align: center;">10</div> <div style="text-align: center;">15</div> </div>	(e,g,h)
<u>H3</u>	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> (Me) minor Ac → (Me) (P) Ala-Arg-Thr-Lys-Glx-Thr-Ala-Arg-Lys-Ser-Ser-Gly-Gly-Lys-Ala-Pro-Arg-Lys-Glx-Leu-Ala-Thr-Lys-Ala-Ala-Arg-Lys-Ser- </div> <div style="text-align: center;">5</div> <div style="text-align: center;">10</div> <div style="text-align: center;">15</div> <div style="text-align: center;">20</div> <div style="text-align: center;">25</div> </div>	(e,g,i-l)
<u>H4</u>	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> (P) Ac Ac Ac Ac (Me) Ac-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-Lys-Arg-His-Arg-Lys- </div> <div style="text-align: center;">5</div> <div style="text-align: center;">10</div> <div style="text-align: center;">15</div> <div style="text-align: center;">20</div> </div>	(c-f,k,m,n)

References for Table 1.2.7

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|------------------------------|--------------------------------|
| (a) Rall and Cole, 1971 | (h) Shlyapnikov et al., 1975 |
| (b) Sautiere et al., 1974 | (i) Brandt et al., 1974 |
| (c) DeLange et al., 1969 | (j) DeLange et al., 1972 |
| (d) Sung and Dixon, 1970 | (k) Honda et al., 1975b |
| (e) Dixon et al., 1975 | (l) Marzluff and McCarty, 1972 |
| (f) Candido and Dixon, 1972a | (m) Dixon et al., 1969 |
| (g) Candido and Dixon, 1972b | (n) Candido and Dixon, 1971 |
-

subfractions were also acetylated, with two unidentified acetylated amino acids (possibly derivatives of arginine) being formed. The third enzyme (A) was more specific for H3 histone when in a crude form, but when purified was more specific for histone H1 than histone H3 which was however also acetylated. It has been suggested (Gallwitz and Sures, 1972) that H3 is the in vivo substrate of enzyme A but that the changed conformation of the histones resulting from the removal of DNA could render them more or less susceptible to the acetylation by different histone transacetylases. Acetylation of residues other than lysine which occurs with the "B" enzymes could be due to loss of specificity of the enzyme upon extraction since only acetyllysine has been detected (see Table 1.2.7) in post-synthetically acetylated histones. A similar situation exists for a methyltransferase which transfers methyl groups to an arginine and not a lysine residue as expected (Gallwitz, 1971a). In addition when tryptic peptides of histones H4 and H3 acetylated in vivo and in vitro with histone transacetylase were compared, the patterns were found to be different which was implied to mean that a special conformation of histones in the DNA-histone complex is required for the precise specificity of the histone transacetylases (Gallwitz and Sures, 1973). A cautionary word on histone transacetylase multiplicity was sounded by a report of Lue et al. (1973) who showed that histone transacetylases can exist in two forms, either in a histone-histone transacetylase complex (acetyltransferase I) or else as histone-free-histone transacetylase (acetyltransferase II). These forms will behave differently with respect to their elution from DEAE columns, as well as upon density gradient ultracentrifugation. For this reason any study

of histone transacetylase should attempt to use histone-free histone transacetylase extracts, especially if substrate specificity is to be investigated.

The histone transacetylases are tightly bound to chromatin (Racey and Byvoet, 1971, 1972; Lue et al., 1973; Candido, 1975; Harvey and Libby, 1976), and specifically acetylate the arginine-rich histones while bound to the chromatin, but after extraction from the chromatin can also acetylate the lysine-rich histones although to a lesser extent than the arginine-rich histones (Racey and Byvoet, 1972). It was suggested (Racey and Byvoet, 1972) that the influence of steric factors in native chromatin is lost upon removal of the enzyme from the chromatin or else a less specific isoenzyme may have been extracted. It was confirmed by Lue et al. (1973) that in vitro acetylation by histone transacetylase (acetyltransferase I and acetyltransferase II) preferentially acetylates H4 and H3 but that other fractions were also acetylated. The differing specificities of acetyltransferase I and acetyltransferase II and the A and B enzymes of Gallwitz and Sures (1972) indicate that these enzymes are different but this is still an unresolved question as a mixture of histone transacetylases would behave in a similar manner to acetyltransferase I or acetyltransferase II (Lue et al., 1973). Candido (1975) studied histone transacetylase in trout testis nuclei and found that H3 and H4 had the highest acetylation followed by H2B and H2A with H1 incorporating no acetyl groups. Nuclear extracts containing histone transacetylase showed a preference for H3 and H4 as substrates with the other fractions being acetylated to a much lesser degree. That histone transacetylase occurs in the chromatin was implied to mean that histones are acetylated after they are incorporated into the deoxynucleoprotein complex and that the biological function of histone acetylation is probably related to structural changes required in the chromatin (Racey and Byvoet, 1972). Genetically inactive sea urchin sperm contain no histone transacetylase which lends support to this idea since sperm chromatin structural changes would only be required after fertilisation and sea urchin eggs have been shown to contain histone transacetylase which could catalyse the acetylation of the sperm chromatin (Sures and Gallwitz, 1975).

Complex formation between histones and acidic proteins may be an inherent feature of nuclear histone transacetylase extracts. Histone

transacetylases have been shown to be acidic proteins (Gallwitz and Sures, 1972) and another nuclear acidic protein, DNA-dependent RNA polymerase (Elgin et al., 1971), was also found in histone transacetylase extracts (Lue et al., 1973; Racey and Byvoet, 1972). The interactions of histone transacetylase with histones as indicated by Lue et al. (1973) may have biological significance since it has been shown that specific histone aggregates occur in solution (Kornberg and Thomas, 1974; Thomas and Kornberg, 1975; Weintraub et al., 1975) which may be important in a repeating chromatin subunit (Kornberg, 1974; Baldwin et al., 1975; Lewin, 1975; van Holde et al., 1975). Since histone acetylation has been correlated with histone synthesis and therefore with the assembly of new chromatin in the model proposed by Dixon's group (which will be discussed in more detail below), specific histone aggregates may be the true substrates for the histone transacetylases (Candido, 1975).

Cytoplasmic histone transacetylases have also been reported. Libby (1968) demonstrated that rat uterus cytosol and nuclei contain an enzyme system which can acetylate histones. Cytoplasmic and nuclear histone transacetylases were prepared from rat liver by Pestana et al. (1971). The nuclear enzyme preferred the arginine-rich histones as substrate while the cytoplasmic histone transacetylases showed an equal affinity for either the lysine- or arginine-rich histones. Calf thymus cytoplasm contains an enzyme (Horiuchi and Fujimoto, 1972) which acetylates mainly H4 histone, forming ϵ -N-acetyllysine in the histones. It has also been shown (Horiuchi and Fujimoto, 1973) that 50-60% of the ^{14}C -acetyl groups incorporated into histone H4 were contained in a thermolysin peptide containing amino acids 10-21 which indicates that lysine₁₂ (and/or lysine₁₆) is the main site of acetylation in H4 by this enzyme (see Table 1.2.7). In contrast to these results, Harvey and Libby (1976) have recently separated three histone transacetylases found in the cytosol of calf endometrium which differ in their pH optima, preferences for histone subfractions as substrates and stability to heat denaturation. Histone transacetylase I showed the greatest preference for histone H2B and H3 as substrates while H2A followed by H2B were the most acetylated by histone transacetylase II and III. The authors do not preclude the possibility that histone transacetylase II and III may be different forms of the same enzyme, analogous to acetyl transferase I and acetyltransferase II of Lue et al., (1973). The function of cytoplasmic histone transacetylase at this

stage is unknown but could be required for the acetylation of newly synthesised histone which occurs in the cytoplasm (Ruiz-Carrillo et al., 1975; Jackson et al., 1976). Another suggestion is that possibly the cytoplasmic enzyme is the precursor of the nuclear enzyme (Horiuchi and Fujimoto, 1972).

Since histone acetyl groups are subject to rapid turnover, a search for the enzymes responsible for acetyl group removal was initiated by Fujimoto and his group who were able to detect an enzyme activity in calf thymus cytoplasm (Inoue and Fujimoto, 1969, 1970), which could remove acetyl groups from histone. Biologically acetylated histone was a better substrate than chemically acetylated histone which indicated that the deacetylase has a high degree of specificity to distinguish specific acetyllysyl groups which are also distinguished by the histone transacetylase molecule (Inoue and Fujimoto, 1970, 1972). The histone deacetylase was found to deacetylate ϵ -N-acetyllysine residues in histones H3 and H4 (Inoue and Fujimoto, 1972). Pronase treatment of histone destroyed its ability to be deacetylated (Inoue and Fujimoto, 1970) which indicates that the enzyme needs a relatively large molecule to act as substrate, a result which was confirmed later (Horiuchi and Fujimoto, 1973; Krieger et al., 1974). Histone deacetylases have been shown to occur in a range of animal and plant tissues (Fujimoto, 1972). When histone H2A was chemically acetylated with acetyl-CoA the acetyl groups were found to be specifically located in the N-terminal region of the molecule (Fujimoto and Segawa, 1973) and to be removed by the calf thymus cytoplasmic histone deacetylase. This was interpreted to mean that H2A could possibly be acetylated and rapidly deacetylated in vivo, however the existence of a H2A-specific histone deacetylase has not been shown. A nuclear histone deacetylase was isolated from calf thymus (Vidali et al., 1972) which specifically deacetylated H3 and H4 by cleavage of ϵ -N-acetyllysine residues. This enzyme was found to be an acidic protein and to be able to bind to histones or chromatin. It is interesting that histone transacetylase and DNA-dependent RNA polymerase have also been shown to be acidic proteins (Gallwitz and Sures, 1972; Elgin et al., 1971) and could together with histone deacetylase and histone form a complex which could be important in transcriptional control. A further study of the substrate specificity of the nuclear enzyme (Krieger et al., 1974), showed that acetylated H4 peptides containing amino acid residues 1-84 and 1-37 were both active as substrate for the histone deacetylase

but that a peptide containing amino acids 15-21 could not serve as substrate even though the major site of acetylation at lysine₁₆ (DeLange et al., 1969), is contained in this peptide. It has also been shown (Horiuchi and Fujimoto, 1973) that an acetylated H4 peptide containing amino acid residues 10-21 cannot be deacetylated by the cytoplasmic enzyme even though this peptide contains two reported sites of acetylation (see Table 1.2.7). A multiplicity of the enzyme was indicated by the elution pattern on DEAE-cellulose or Sepharose (Kikuchi and Fujimoto, 1973; Inoue and Fujimoto, 1970) as well as the different substrate specificities of the peaks obtained. These enzymes were unable to deacetylate chromatin-bound histone (Kikuchi and Fujimoto, 1973). Kaneta and Fujimoto (1974) have confirmed the existence of a nuclear calf thymus histone deacetylase and found that this enzyme can deacetylate chromatin-bound histone and differs from the cytoplasmic enzyme in its chromatographic behaviour. The presence of at least two forms of histone deacetylase with differing substrate specificities towards either chromatin-bound or free histone is indicated. It is possible that "free-histone-deacetylase" is a precursor of "chromatin-bound-histone-deacetylase" and that "free-histone-deacetylase" can deacetylate chromatin-bound histone after forming a complex with exogenous histone (Kaneta and Fujimoto, 1974).

Histone modification has been suggested by Dixon's group (Louie et al., 1973; Dixon et al., 1975e; Dixon, 1976) to be involved in (a) the attachment of newly synthesised histones to DNA; (b) changes in chromosomal coiling; (c) changes in chromosomal activity during the cell cycle or differentiation; (d) removal of histones from DNA (particularly the transient removal of histones around the replication fork during DNA synthesis); or (e) maintenance of the correct conformation to DNA. These authors have also suggested that cytoplasmic or nuclear modifications could occur either (i) shortly after synthesis of the "new" histone while it is free in solution and passing from the site of synthesis on the cytoplasmic ribosomes (Borun et al., 1967) to the site of chromatin assembly in the nucleus; or (ii) later after the chromatin has been assembled and a change of physiological state requires that the conformation of the basic regions of histones bound to DNA is altered (Louie et al., 1973; Dixon et al., 1975f). This possibility is supported by histone transacetylase and histone deacetylases having been shown (see above) to occur in both the nuclei and cytoplasm. Histone acetylation

could be involved in all of the above functions. The involvement of histone acetylation in the assembly or disassembly of the nucleosome is also possible (Dixon, 1976), particularly as histone transacetylase and histone deacetylases have been shown to be acidic proteins (Vidali et al., 1972; Gallwitz and Sures, 1972) which can bind electrostatically to histones.

It should be emphasised that the possible functions for histone modification given above are not mutually exclusive and that histone acetylation for example could be involved in any or all of these functions.

A well defined and detailed model for the involvement of phosphorylation and acetylation in the binding of newly synthesised histones H2A and H4 to DNA during trout spermiogenesis proposed by Dixon's group (Louie et al., 1973) is discussed below.

When the basic N-terminal region of histones H4 and H2A (which show strong sequence homology) is in an α -helical conformation it can fit into the major groove of DNA with the appropriate ionic interactions between the basic amino acid residues and the DNA phosphate groups. However, charged amino acid side chains are α -helix destabilisers, thus if a correct conformation of the H2A and H4 histones when complexed to DNA is α -helical, a mechanism for attaining this conformation has to be postulated. Dixon and his group have suggested that the acetylation of H4 occurring at lysine residues -5, -8, -12 and -16, and acetylation of lysine-5 and phosphorylation of serine-1 occurring in H2A, may be involved in the achievement of the correct binding conformation of histones to DNA. The sequential acetylation of H4 may neutralise the positive lysyl charges and allow the histone to assume the correct conformation, presumably an α -helix, to fit into the major groove of DNA. Deacetylation can then expose the positive charges again and lock the histone into the DNA via ionic linkages. Similarly the phosphorylation and acetylation of H2A may induce the NH_2 -terminal region to assume an α -helical conformation and after the correct binding has occurred demodification could follow. (It is possible that when RNA transcription is required that acetylation (and phosphorylation) could occur to loosen the DNA-histone binding to allow the passage of the replication fork, followed by demodification to again lock the histones into place (Dixon, 1976)). Dixon suggests that the histones are bound non-specifically to the naked new DNA double helix

and then are induced to assume the correct conformation. It is not known at this stage whether "old" histones remain with the "old" strands of DNA or not as conflicting reports exist. Tsanev and Russev (1974) suggest that "old" histones stay with the "old" strands during replication, while Jackson et al. (1975) reported that "new" histones are randomly deposited onto the chromosome. It is not known at this stage which histones in the nucleosome complex would go to either strand if semi-conservative deposition of histones occurs. Louie et al. (1973) suggest that the acetylation of specific lysyl residues in the DNA-bound histones could loosen the binding of the N-terminal region and allow the conformation of the histones to change which may allow the histone to be removed. However, these authors stress that the exact mechanism of histone removal, whether by enzymatic modification or proteolysis or both, is unknown.

A recent report (Ruiz-Carrillo et al., 1975) has investigated the processing of newly synthesised histones in duck erythrocytes. These authors did not find the degree of multiple acetylation of "new" histones required by Dixon's model but instead found transient cytoplasmic phosphorylation (at Ser-1) and acetylation (at undetermined lysine residues) of H4 which occurred immediately after synthesis. After the histones have entered the nucleus, an unknown proportion of the histone H4 molecules are rapidly dephosphorylated and/or deacetylated. Later nuclear modifications of H4 include the stepwise acetylation of specific lysine residues as well as phosphorylation. It has been suggested by Ruiz-Carrillo et al. (1975) that the early modifications may be involved in the stepwise assembly of chromatin. The transient forms of H4 may bind to DNA in a manner that favours the co-operative interaction with other histones to form a multimeric histone complex together with the enveloping DNA strand (Ruiz-Carrillo et al., 1975). At this stage it is not known whether the non-histone proteins capable of recognising the modified forms of histones (e.g. histone transacetylases and histone de-acetylases) may also play a part in the correct spacing and assembly of histone complexes on the DNA. It is possible that the histone transacetylase for example may become bound to the histone before the histone-DNA complex is finally formed, as otherwise these enzymes may encounter serious problems of access to their substrate as they approach their sites of reaction once the histone is bound to DNA (Dixon et al., 1975). It is striking that H3 and H4 which are evolutionarily the most conserved histones are those which are subject to the most post-synthetic modifications. It may be

that these histones are recognised by the histone modifying enzymes during chromatin assembly which would also impart limitations on sequence variations on these histones (Smith, 1975).

To summarise the present knowledge of post-synthetic histone modifications one may agree with Dixon that "clearly we are only at the beginning of our understanding of the delicate and subtle effects that such modifications can have on the structure and function of chromatin as it is coiled, uncoiled, replicated, reassembled and its genetic information selectively expressed" (Dixon et al., 1975f).

PART 2

HISTONE TRANSACETYLASES IN RAT LIVER NUCLEI

2.1 INTRODUCTION

Various histone transacetylase (EC 2.3.1) enzymes have been prepared from nuclei, chromatin or cytoplasm and characterised with respect to their kinetic parameters, physico-chemical properties and affinity to the various histone subfractions (see 1.2.7). However nothing is known about the actual sites acetylated in vitro in the various histone molecules by these histone transacetylase preparations. This study investigated the sites of acetylation of histones by an extract enriched in histone transacetylase activity which was used to catalyse the formation of ^3H -acetyl histone, from which histone subfractions were prepared and subjected to Edman degradation.

Since the histone transacetylase was being used merely as a tool to study the sites acetylated, no attempt was made to purify the enzyme to homogeneity. Neither was a detailed study of the intranuclear localisation of the enzyme nor of its physico-chemical properties undertaken. However, to determine the optimal incubation conditions for the acetylation of histones, a study of some of the properties of the histone transacetylase reaction was done. The substrate specificity of the histone transacetylase extract towards proteins other than histones, total histone preparations isolated by different methods, chromatin bound histones, as well as pure histone subfractions was also studied.

RESULTS :2.2 PREPARATION OF HISTONE TRANSACETYLASES2.2.1 Preparation of crude histone transacetylase extract

A crude histone transacetylase extract was prepared from rat liver nuclei

(4.4.1) by the method described in 4.4.2.

2.2.2 Sucrose gradient ultracentrifugation of crude histone transacetylase extract

Histones bound to histone transacetylase can be removed by treatment with 2 M NaCl (Lue et al., 1973). Crude extract (4.4.2) was run on 5-20% sucrose gradients (4.4.3) containing 0.01, 0.3 or 3.0 M NH_4Cl - TEM. Fig. 2.2.2.1 shows that the distribution of histone transacetylase activity moved significantly towards the top of the gradient when the NH_4Cl concentration was increased from 0.01 to 0.3 M. A further increase to 3 M NH_4Cl had no additional effect. In view of this sucrose gradients were done at 0.3 M NH_4Cl in an SW 40 rotor because of its higher capacity (Fig. 2.2.2.2).

A summary of the purification is shown in Table 2.2.2.1.

TABLE 2.2.2.1

SUMMARY OF PURIFICATION OF HISTONE TRANSACETYLASE

Fraction	Specific activity (units/mg)	Total activity (units)	mg protein	Purification in step (-times)	Overall purification (-times)	Recovery in step (%)
Nuclei	0.3	91.5	305	1	1	-
Crude extract	3.5	248	71	12	12	>100
Sucrose gradient fractions	33.3	532	16	9.5	114	>100

(1 unit enzyme activity : 1 pmole acetate incorporated under the incubation conditions used (4.3). Protein concentration was determined spectrophotometrically (4.2.1)).

The more than 100% recovery of activity in the crude extract is possibly due to the fact that upon solubilisation of the enzyme it has a much greater access to substrate than when it is bound in the nucleus. The

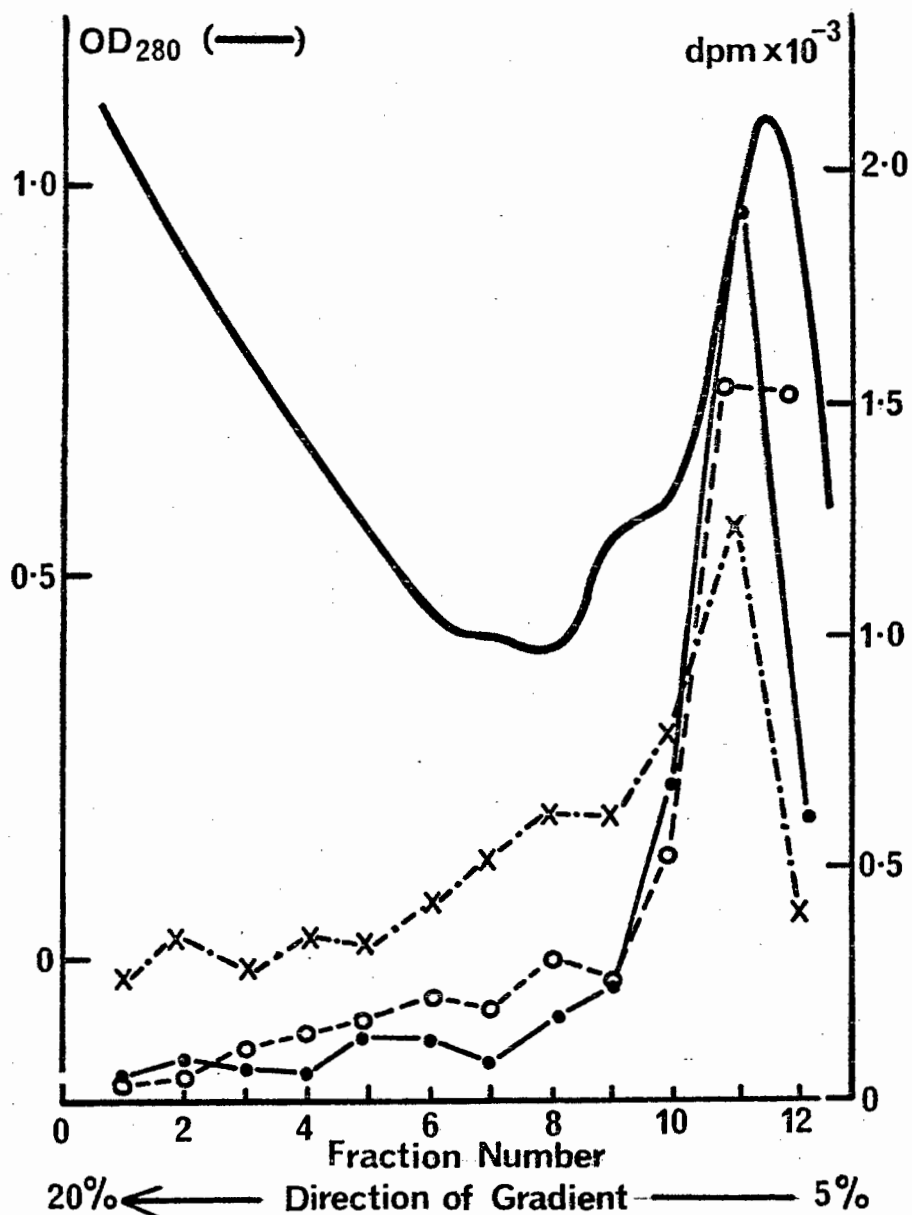


Fig. 2.2.2.1 : Effect of $[\text{NH}_4\text{Cl}]$ on ultracentrifugation of histone transacetylase.

- OD_{280}
 -x-x-x Histone transacetylase activity from 0.01 M NH_4Cl gradient
 -o-o-o Histone transacetylase activity from 0.3 M NH_4Cl gradient
 -●-●-● Histone transacetylase activity from 3.0 M NH_4Cl gradient

Equal aliquots of crude extract (4.4.2) were applied to 5-20% sucrose gradients containing 0.01, 0.3 or 3.0 M NH_4Cl -TEM and centrifuged for 3½ h at 420 000 g in a SW 65Ti (Beckman) rotor (4.4.3).

Histone transacetylating activity was assayed as described in 4.3.

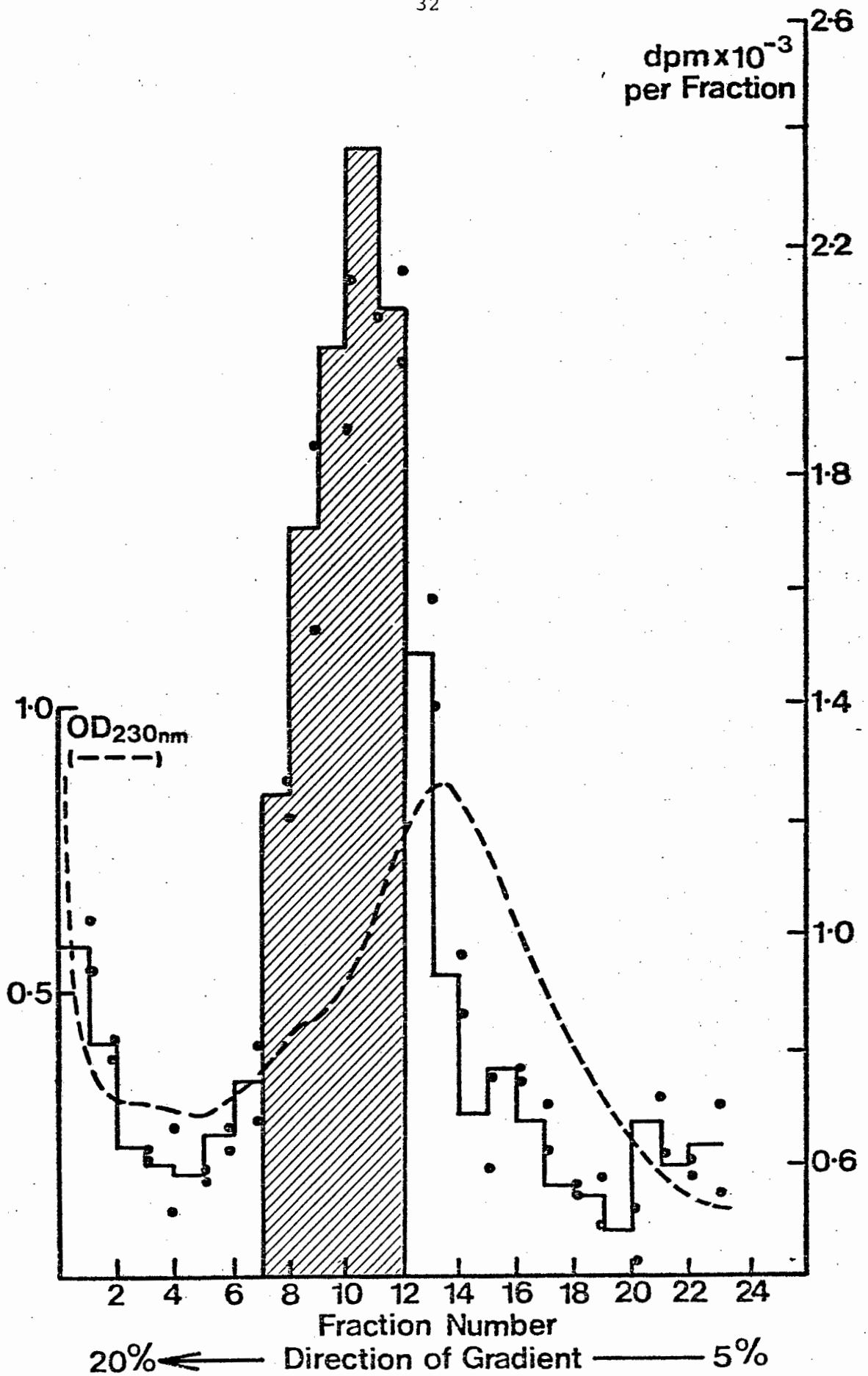



Fig. 2.2.2.2 : Sucrose gradient centrifugation of crude histone transacetylase. Aliquots (600 μ l) of the crude extract of histone transacetylase were applied to 5-20% sucrose gradients in 0.3 M NH_4Cl -TEM and subjected to centrifugation at 284 000 g for 30 h in an SW 40 rotor (4.4.3). Histone transacetylating activity (●—●) was assayed as described in 4.3.  Pooled histone transacetylase fractions (sucrose gradient fractions).

sucrose gradient step could result in the removal of an inhibitor of the histone transacetylase (or maybe of a deacetylase even) which could explain the more than 100% recovery of activity in this step.

2.2.3 Further purification of sucrose gradient fractions

2.2.3.1 Ion exchange chromatography on DEAE-cellulose

Ion exchange chromatography was done as described in 4.4.4. Fig. 2.2.3.1 shows that a single peak of histone transacetylase activity was found eluting at 0.11 M NH_4Cl .

As can be seen from Table 2.2.3.1, ion exchange resulted in a further purification (3 times) of the histone transacetylase, but a low yield (15%) and with such poor stability upon storage (2.5) that this procedure was not routinely used for histone transacetylase preparation.

2.2.3.2 Gel filtration of sucrose gradient fractions

When gel filtration of sucrose gradient fractions was carried out in 0.3 M NH_4Cl -TEM on Sepharose 4B or Sephadex G-200 (column dimensions 1.5 x 30 cm), a single peak of histone transacetylase activity was found which eluted at the breakthrough volume in the case of Sephadex G-200 or else in the inner volume of Sepharose 4B. Table 2.2.3.1 shows the purifications and yields obtained using these procedures. The further purification obtained was not considered economical when the percentage yields and time involved in this procedure on a routine basis were considered. Thus the sucrose gradient fractions were routinely used as the histone transacetylase enzyme source for further investigations.

Though ion exchange chromatography on DEAE-cellulose or gel filtration gave further purification but decreased yields (see Table 2.2.3.1), sucrose gradient fractions were used as the enzyme source for further investigations, because when the sucrose gradient fractions were freeze dried and subjected to polyacrylamide gel electrophoresis (4.2.5), these fractions were found to be histone-free, an essential prerequisite in a study of histone transacetylase substrate specificity.

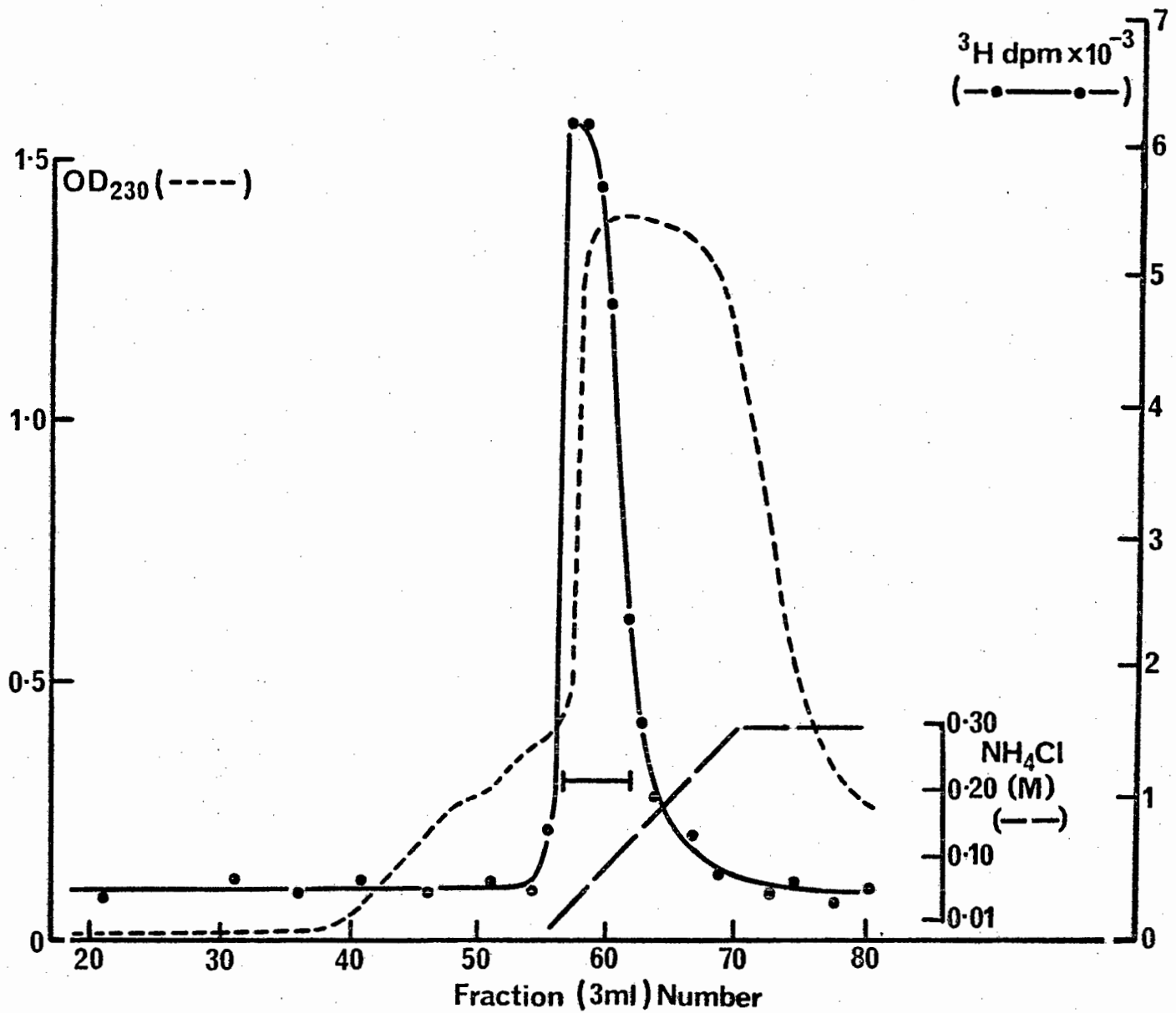


Fig. 2.2.3.1 : DEAE-cellulose chromatography of sucrose gradient fractions. Elution was performed as described in 4.4.4 using a linear 0.01 to 0.3 M NH₄Cl gradient in TEM. Histone transacetylase activity (●—●) was assayed as described in 4.3.

TABLE 2.2.3.1

PURIFICATION OF SUCROSE GRADIENT FRACTIONS
BY ION-EXCHANGE- OR GEL FILTRATION CHROMATOGRAPHY

Fraction	Specific activity (units/mg)	Total activity (units)	mg protein	Purification obtained	Recovery (%)
Sucrose gradient fractions	33.3	532	16	1	-
DEAE-cellulose fractions	100.0	80	0.8	3	15
G-200 fractions	81.8	270	3.3	2.4	51
Sephadex 4B fractions	55.9	162	2.9	1.6	30

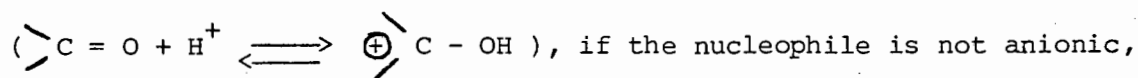
(Unit of enzyme activity is defined in footnote to Table 2.2.2.1. DEAE-cellulose chromatography was performed as described in 2.2.3.1. and 4.4.4. Gel filtration on Sephadex G-200 or Sephadex 4B was done as described in 2.2.3.2. Histone transacetylase activity was assayed as per 4.3).

2.3

NON-ENZYMATIC ACETYLATION

It was consistently observed that acetyl groups were transferred to histones in the absence of enzyme. This confirmed the observations of previous workers (Gallwitz and Sekeris, 1969; Paik et al., 1970; Gallwitz, 1970a; Racey and Byvoet, 1971; Gallwitz and Sures, 1971) who also observed that this acetylation increases with increasing pH. The activity incorporated amounted to approximately 200-300 dpm under the conditions of the assay. Such incorporation could either be due to non-enzymatic acetylation or the presence of an acetyltransferase contaminant in the histone preparations used as substrate.

In $\text{CH}_3 - \overset{\text{O}}{\underset{*}{\text{C}}} - \text{S} - \text{CoA}$ the carbonyl carbon (C_*) is strongly positive and can be nucleophilically attacked by the lone pair of electrons on $\epsilon\text{-}\ddot{\text{N}}\text{H}_2$ -protein in histones. For interpreting acetyl transfer from AcCoA to the nucleophilic agent ($\epsilon\text{-}\ddot{\text{N}}\text{H}_2$ of histone) it is not even necessary to envisage delocalisation of the π electrons of the $\text{C}=\text{O}$ bond as even without this, the carbon (C_*) of the acetyl group has sufficient positive charge to interact with the free electron pair of the $\epsilon\text{-}\text{NH}_2$ group with subsequent cleavage of the C-S bond. In dilute aqueous solution at $20\text{-}40^\circ\text{C}$ acyl thiols in fact have a specific affinity for amines, e.g. $\epsilon\text{-}\text{NH}_2$ of histones (Jaenicke and Lynen, 1960). The strong pH dependence of the reaction (Gallwitz and Sekeris, 1969; Paik et al., 1970; Racey and Byvoet, 1971) is due to the fact that although nucleophilic attack on the carboxyl carbon is facilitated by protonation



e.g. $\text{R-}\ddot{\text{N}}\text{H}_2$, any quantity of acid converts it to the unreactive species $\text{R-}\overset{+}{\text{N}}\text{H}_3$ (Sykes, 1966). From these considerations it is obvious that non-enzymatic acetylation is an inherent property of the incubation system.

2.3.1 Effect of temperature on non-enzymatic acetylation

To confirm that the acid-extracted total histone (4.5.1) used as substrate is not contaminated with histone transacetylase, the temperature dependence of acetyl transfer in the absence of the enzyme preparation was determined.

Fig. 2.3.1 shows that the acetylation increased with increasing temperature up to 75° which suggests that this is a non-enzymatic phenomenon since "the rate of inactivation of enzymes in solution increases rapidly with temperature and in nearly all cases inactivation becomes virtually instantaneous well below 100° and in the majority of cases below 70° " (Dixon and Webb, 1964). The decrease in acetylation in going from 75° to 90° is probably due to decomposition of AcCoA at the higher temperature. Further support for the incorporation being non-enzymatic is that the histone substrate is exposed to 0.25 M HCl followed by freeze-drying during preparation which would be expected to inactivate the majority of enzymes. The histone transacetylating activity in the crude extract (2.2.1) is very sensitive to temperature with 93% of the activity being lost if the preparation is heated for 15 min at 60° before incubation.

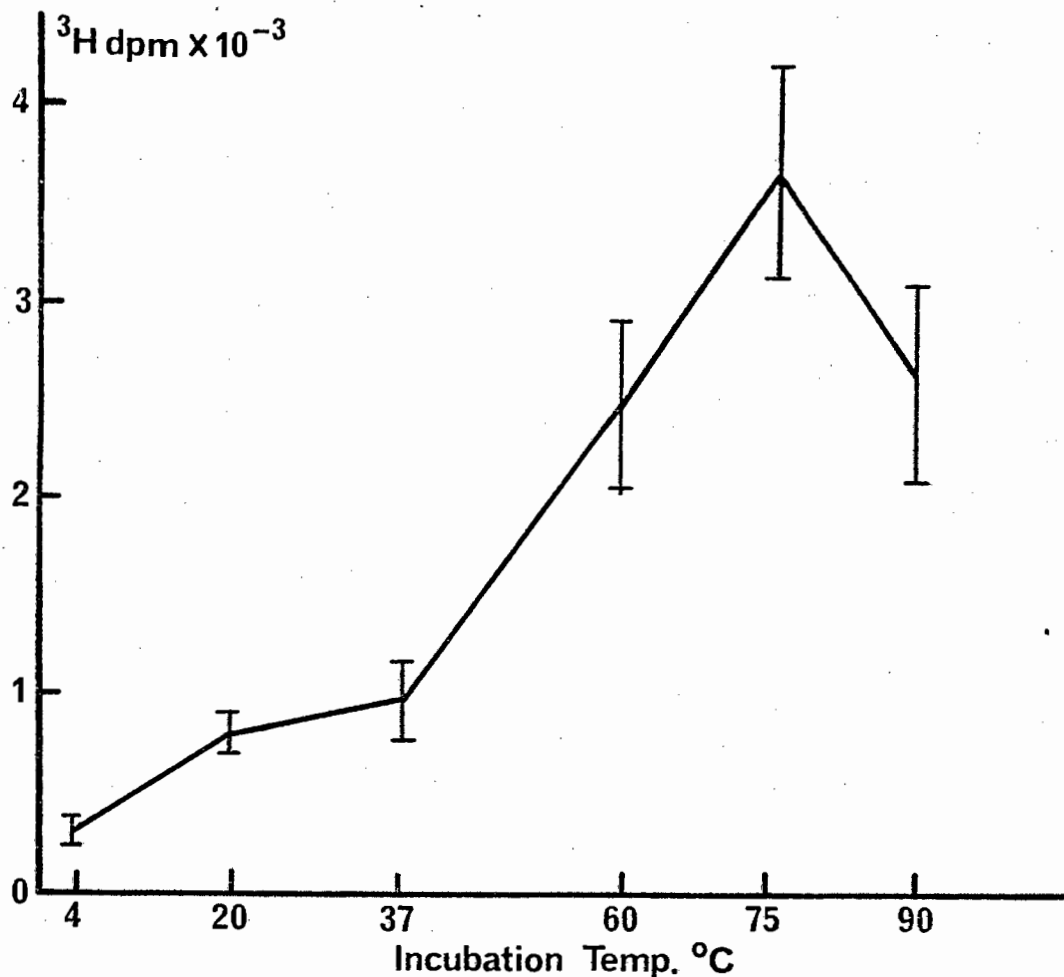


Fig. 2.3.1 : Effect of incubation temperature on histone acetylation in the absence of histone transacetylase extract. Temperatures of incubation were 4, 20, 37, 60, 75 and 90° with each temperature being assayed in quadruplicate. 0.3 M NH_4Cl -TEM and MKT buffer were mixed in the ratio of 4:1 (v/v) and the pH at 4°C adjusted to 7.9. The solution was then heated, and at the temperatures indicated above, aliquots were removed for incubation (after first adjusting the pH to 7.9 with 0.25 N NaOH) with 0.08 ml of a solution containing 1 mg of acid extracted total histone and 0.02 μCi of ^3H AcCoA for 30 min at the temperatures to which the aliquots had been heated. At the end of the incubation the histones were isolated and counted as described in Materials and methods (4.3).

In view of these findings the acetylation in the buffer blanks is concluded to be non-enzymatic in nature rather than due to a contamination of the histone substrate with histone transacetylase. All assays were therefore accompanied by corresponding buffer blanks to correct for non-enzymatic acetylation.

2.4

SOME PROPERTIES OF HISTONE TRANSACETYLASE

In order to be able to prepare ^3H -acetyl histones with a high enough specific activity and yield to enable sequencing studies to be undertaken (Part 3) it was necessary to determine the optimal conditions for acetylation in the incubation system used.

2.4.1

Effect of ionic strength on histone transacetylase

When nuclei were assayed for histone transacetylase activity it was found that an ionic strength above 0.18 causes almost total loss of histone transacetylase activity (Fig. 2.4.1.1). In subsequent experiments all histone transacetylase assays were done at an ionic strength of 0.122.

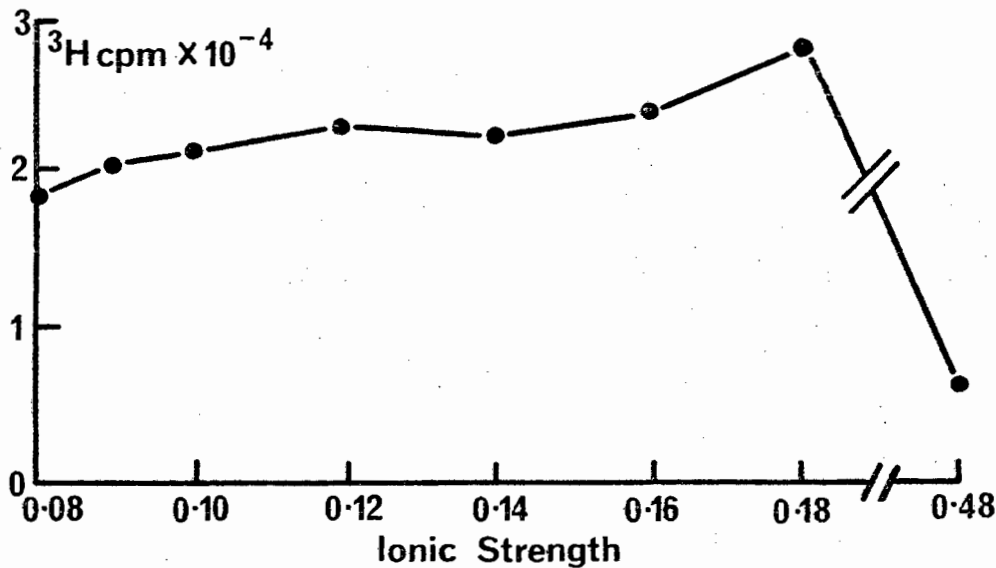


Fig. 2.4.1.1 : Effect of ionic strength on histone transacetylase activity in nuclei. Nuclei corresponding to 0.5 g fresh liver were suspended in 0.5 ml of 0.25 sucrose-TEM which was adjusted to different ionic strengths by the addition of 5 M NaCl. Histone transacetylase activity was assayed by incubation at 37° for 30 min using 1 μCi of ^3H AcCoA (4.3). The reaction was terminated and histones recovered and counted as described in 4.3.

2.4.2 Effect of pH on histone transacetylase

Histone transacetylase showed a pH optimum around 8.0 (Fig. 2.4.2.1). To keep non-enzymatic acetylation (2.3) at a minimum, pH 7.9 was used in all subsequent assays.

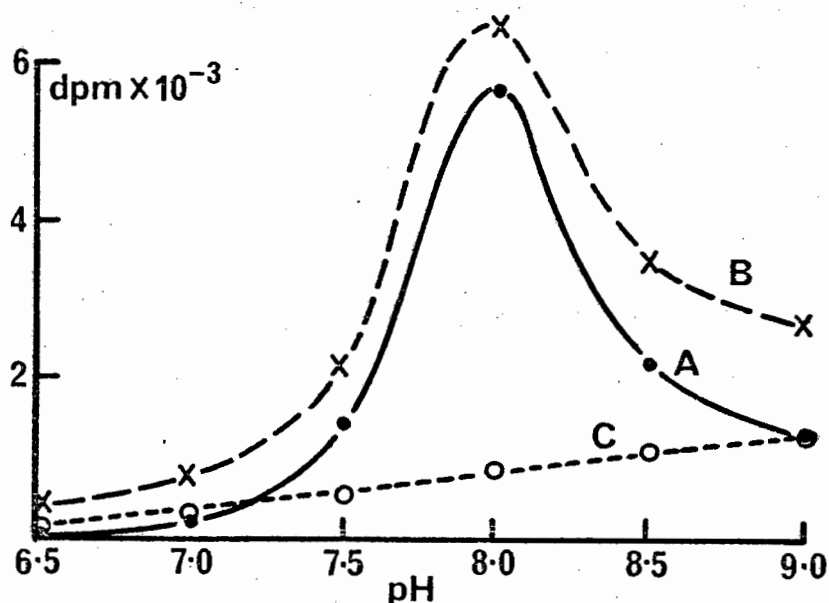


Fig. 2.4.2.1 : Effect of pH on histone transacetylase activity of DEAE fractions. Aliquots of DEAE-cellulose fractions were incubated with ^3H -AcCoA as described in 4.3, except that the pH was adjusted before incubation. Histones were recovered as described in 4.3.

- A Histone transacetylase activity of DEAE fractions corrected for non-enzymatic acetylation
- B Histone transacetylase activity of DEAE fractions not corrected for non-enzymatic acetylation
- C Histone transacetylase activity of buffer blanks (non-enzymatic acetylation).

2.4.3 Effect of incubation time on histone transacetylase

When sucrose gradient fractions were incubated for up to 120 min the reaction was complete by 30 min (Fig. 2.4.3.1).

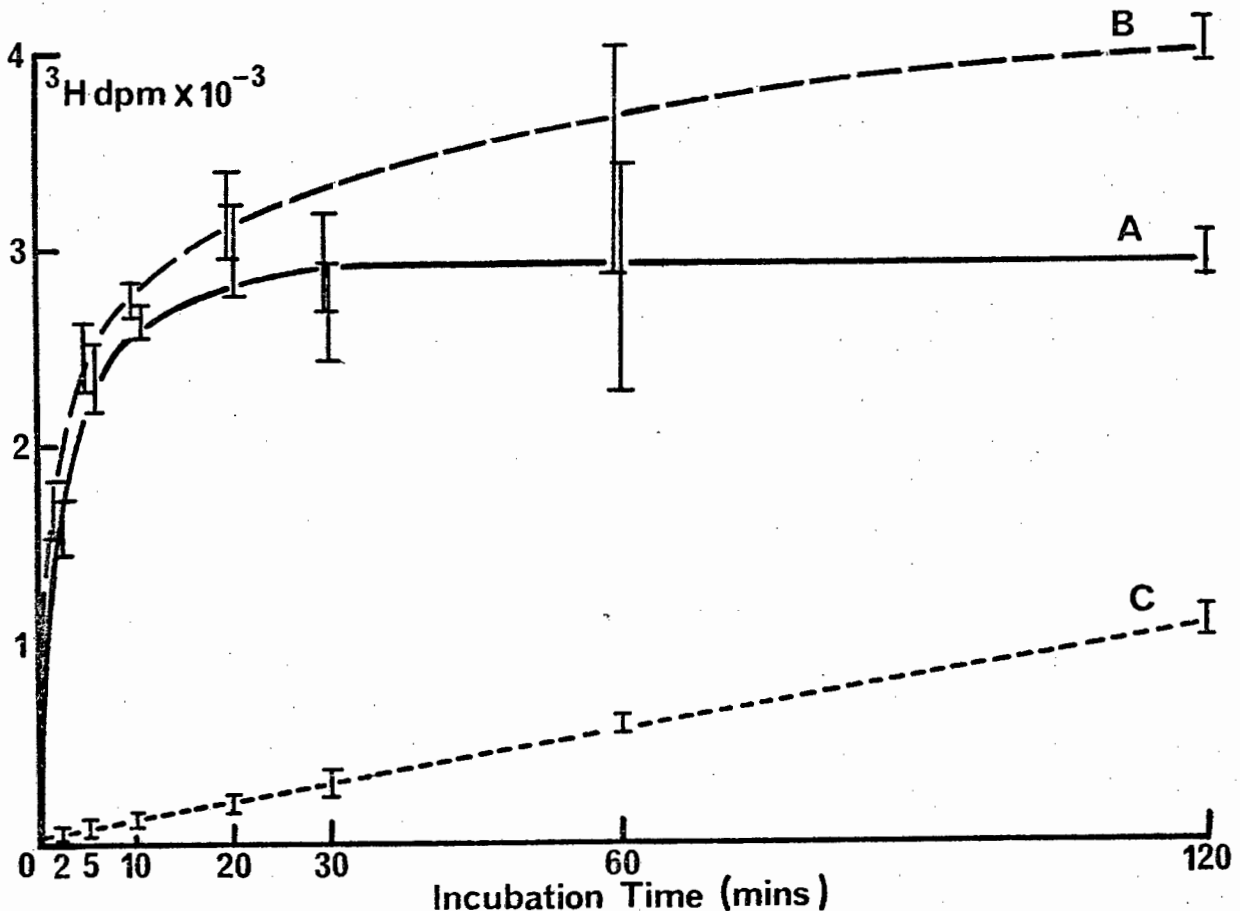


Fig. 2.4.3.1 : Effect of incubation time on histone transacetylase activity of sucrose gradient fractions. Sucrose gradient fractions were incubated as described in 4.3 with the exception that 40 μ l of sucrose gradient fraction rather than 13 μ l, and 50 μ l of 0.3 M NH_4Cl -TEM rather than 78 μ l were incubated with the other constituents. Incubation conditions and histone isolation and counting were performed as described in 4.3 for varying incubation times. Buffer blanks were also incubated to correct for non-enzymatic acetylation.

- A Histone transacetylase activity of sucrose gradient fractions corrected for non-enzymatic acetylation
- B Histone transacetylase activity of sucrose gradient fractions not corrected for non-enzymatic acetylation
- C Histone transacetylase activity of sucrose gradient buffer blanks (non-enzymatic acetylation).

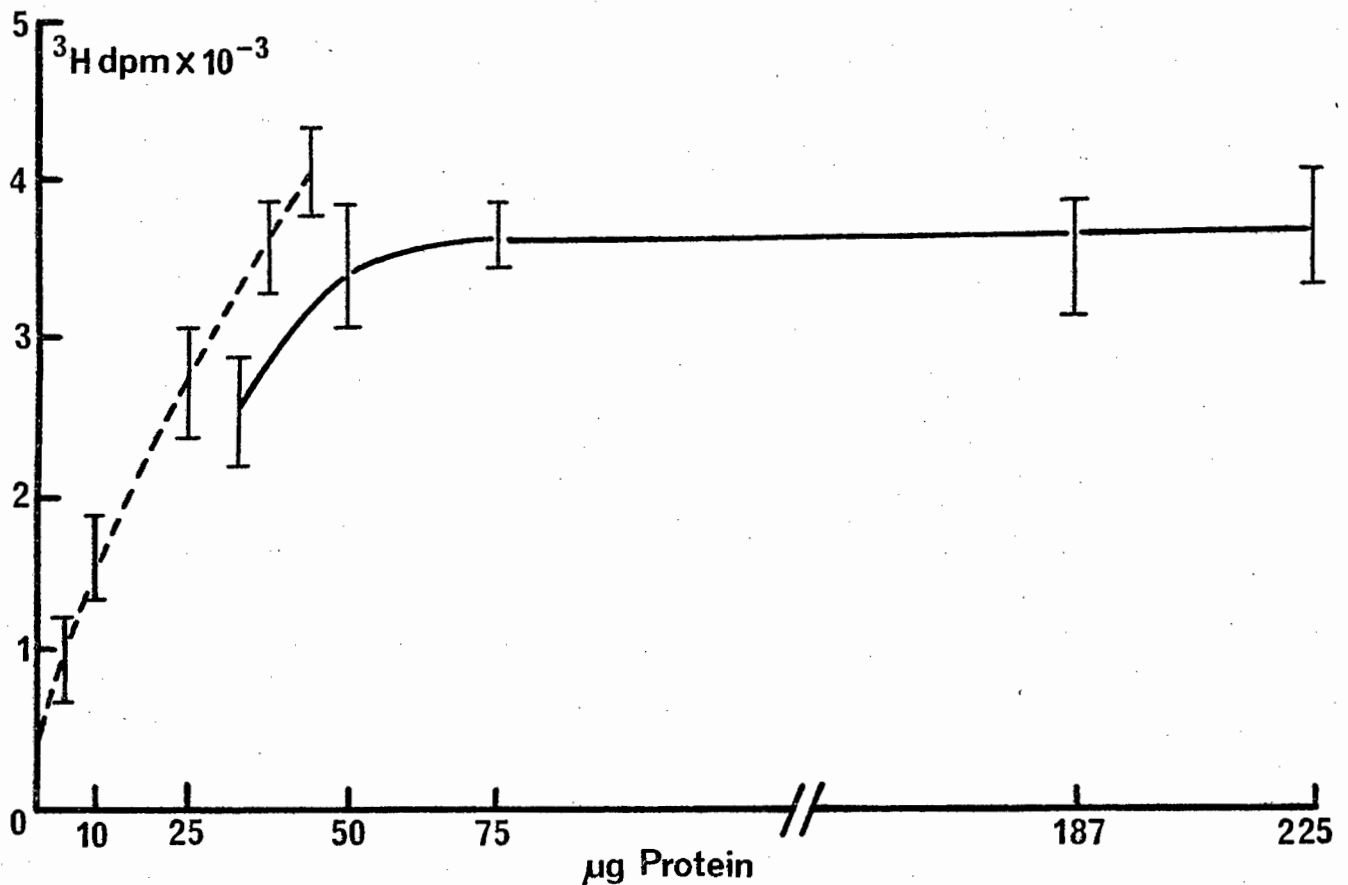


Fig. 2.4.4.1 : Effect of enzyme concentration on histone acetylation by sucrose gradient fractions. Aliquots of sucrose gradient fractions were incubated as described in 4.3 with 1 mg acid extracted total histone for 30 min at 37°C. Up to 90 μ l of sucrose gradient fractions with a protein concentration of 0.5 mg/ml or else sucrose gradient fractions which had been concentrated (5 x) by dialysis against a 75% polyethylene glycol (PEG) solution in 0.3 M NH_4Cl -TEM to a protein concentration of 2.5 mg/ml were incubated.

———— Concentrated sucrose gradient fractions
 ----- Unconcentrated sucrose gradient fractions

2.4.4 Effect of enzyme concentration

Increasing the amount of enzyme protein beyond 45 μ g (90 μ l) did not result in a further increase in histone acetylation (Fig. 2.4.4.1). Dialysis resulted in the loss of about 20% of the histone trans-acetylase activity (Fig. 2.4.4.1). 45 μ g of enzyme protein (undialysed) was used in all subsequent experiments.

2.4.5 Effect of histone substrate concentration on histone transacetylase

Total acetyl incorporation could not be increased by raising the histone concentration beyond 1.72 mg/ml, i.e. 1 mg histone in incubation (Fig. 2.4.5.1). The specific activity of the product decreased with increasing substrate concentration. As a high specific activity as well as a reasonable yield of ^3H -acetyl total histone is required for sequencing studies (Part 3) it was decided to use an enzyme:histone ratio of approximately 1:10 (i.e. 45 μg enzyme protein + 500 μg histone substrate) at which 91% of maximal total incorporation is being achieved with a sufficiently high specific activity.

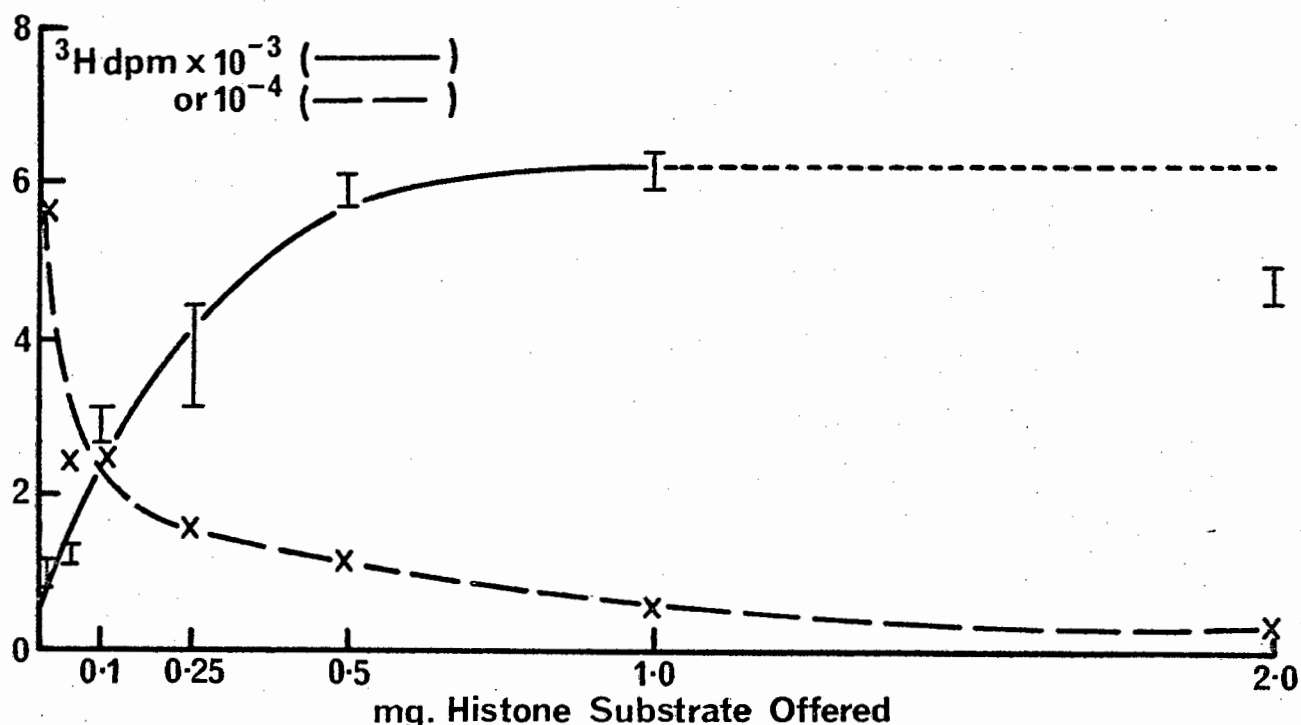


Fig. 2.4.5.1 : Effect of histone substrate concentration on histone acetylation by sucrose gradient fractions.

— Total dpm incorporation into histone offered
 -x--x- dpm/mg histone

Incubations were performed as described in the text (2.4.5) and in Materials and methods (4.3), except that 90 μl (45 μg) of sucrose gradient fractions were used and after termination of the reaction by acidification (4.3), carrier total histone was added to 2 mg. The samples were processed and histones counted as described in 4.3.

2.4.6

Effect of ^3H -acetyl CoA concentration

The optimal acetyl CoA concentration was determined by varying the concentration of AcCoA between 0.033 and 11.4 μM (Fig. 2.4.6.1). Under these conditions an apparent K_M value of 1.2×10^{-6} M and V_{\max} of 1.4×10^6 dpm (62 pmole acetate incorporation) was found. The apparent K_M and V_{\max} values were calculated from the data used to plot Fig. 2.4.6.1 using a Wang computer program (Jost, 1970).

The low level of incorporation of acetyl groups (3.6 mmole/mole) is discussed below (3.6).

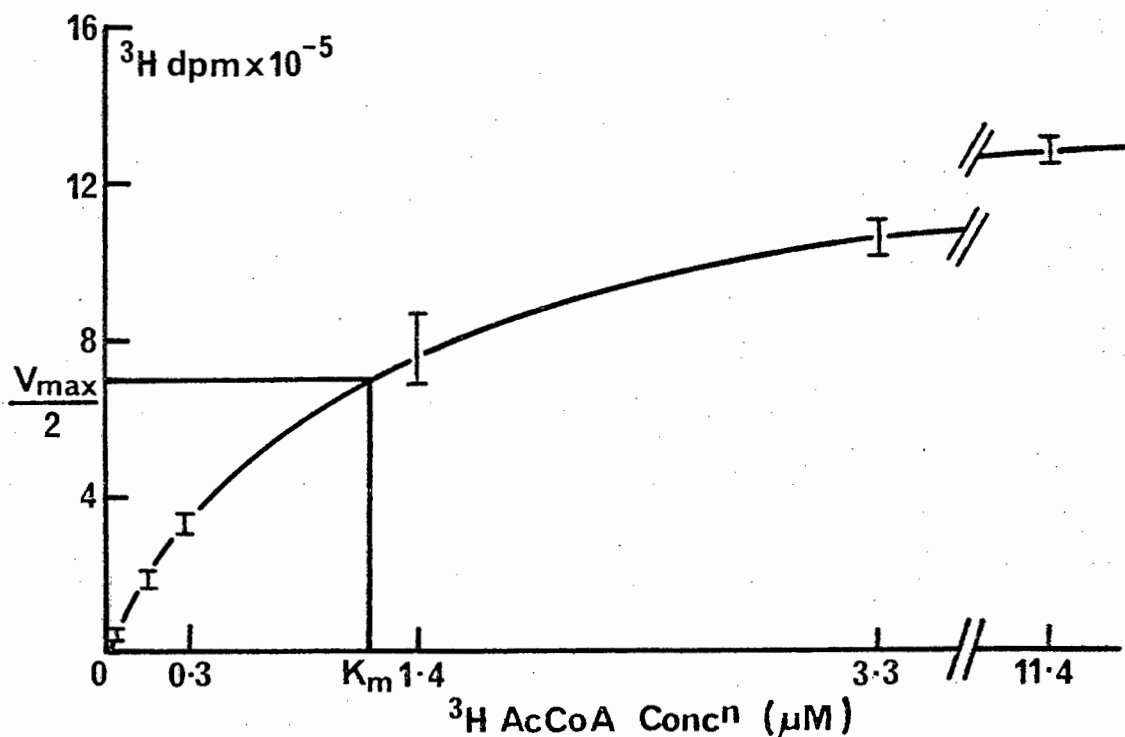


Fig. 2.4.6.1 : Effect of ^3H -AcCoA concentration on histone acetylation. Sucrose gradient fraction (45 μg enzyme protein) was incubated with 250 μg of total histone as described in 4.3 except that the AcCoA concentration was varied between 0.033 and 11.4 μM .

2.5

STORAGE OF HISTONE TRANSACETYLASE PREPARATIONS

The stability of DEAE and sucrose gradient preparations was investigated. As Table 2.5.1 shows the DEAE fractions lost 61% of initial activity after only 10 days of storage; whereas the sucrose gradient fractions are stable for at least 8 days. Sucrose gradient fractions were used within one week of isolation if required for further studies.

TABLE 2.5.1

STORAGE OF DEAE-CELLULOSE AND SUCROSE GRADIENT FRACTIONS

Histone transacetylase preparation	Days of storage	dpm incorporation	%
DEAE-cellulose fractions	0	2299	100
	3	1701	74
	5	1489	65
	10	898	39
Sucrose gradient fractions	0	1560	100
	1	1474	94
	3	1622	104
	5	1579	101
	8	1474	94

Fractions were stored in liquid nitrogen (4.4.5) for the times indicated. 0.4 ml aliquots of the DEAE fractions or 20 μ l of the sucrose gradient fractions were assayed for histone transacetylase activity as described in 4.3.

2.6

SUBSTRATE SPECIFICITY OF HISTONE TRANSACETYLASE

The histone transacetylase preparation was tested for its substrate specificity towards proteins other than histones, total histone prepared by various methods, chromatin-bound histone and electrophoretically pure histone subfractions.

Since histones were shown to be the preferred substrate for the enzyme preparation (Table 2.6.1), various histone preparations were isolated (4.5) and tested for susceptibility to the histone transacetylase.

TABLE 2.6.1

SUBSTRATE SPECIFICITY OF CRUDE EXTRACT (2.2.1)
WITH NON-HISTONE PROTEINS

Substrate	dpm incor- porated	pmole acetate incor- porated	%
Total histone (acid extracted)	5009	2.21	100
Egg white lysozyme	672	0.30	13
α -Chymotrypsin	556	0.25	11
Bovine trypsin	723	0.32	15
Crystalline bovine albumin	590	0.26	12
Crystalline insulin	544	0.24	11
Pancreatic RNase	518	0.23	10
Horse hemoglobin	469	0.21	10
Horse cytochrome C	806	0.36	16
Salmine (histone free)	672	0.30	14
Crude DNase	625	0.28	13
Ovalbumin	703	0.31	14

Crude histone transacetylase extract was incubated as described in Materials and methods (4.3) except that 1 mg of the various protein substrates instead of histone were added. No correction has been made for non-enzymatic acetylation (2.3) of any of these substrates.

Optimal incubation conditions for the enzymatic acetylation of histones have been determined in 2.4 (i.e. ionic strength of 0.122, pH 7.9, incubation temperature of $+37^{\circ}\text{C}$, 30 min incubation time, 90 μl of sucrose gradient histone transacetylase preparation (containing 45 μg protein) as enzyme source and 500 μg total histone substrate). Although the apparent K_m value for ^3H AcCoA has been determined to be $1.2 \times 10^{-6} \text{ M}$ (0.73 μCi

of ^3H AcCoA with specific activity 1.03 Ci/mmol), in testing the susceptibility of various histone preparations to acetylation by the histone transacetylase, only 0.02 μCi of ^3H AcCoA was used since the relative susceptibility of the substrates rather than optimal incorporation of ^3H -acetyl groups was being evaluated.

Calf thymus total histone (500 μg) (4.5.1) was assayed as a substrate in this experiment (Table 2.6.2.I) since it was used previously. Because the histone transacetylase was isolated from rat liver, 500 μg of rat liver total histone (acid extracted) (4.5.3) was also tested as substrate (Table 2.6.2.II).

To substantiate the suggestion that specific histone aggregates might be true substrates for histone transacetylase (Candido, 1975), rat liver protamine displaced total histone was isolated (4.5.4) and 500 μg incubated with the histone transacetylase (Table 2.6.2.III).

The substrate most closely resembling that existing in vivo is isolated chromatin. It has been shown (de Pomerai et al., 1974) that chromatin varies in its template properties depending on the method of preparation. For this reason a rat liver chromatin gel was prepared as described by van der Westhuyzen (1973). This author has shown previously that such chromatin gels retain the biological characteristics of the nuclei from which they were isolated if RNA synthesis is used as an indicator (van der Westhuyzen, 1973). It was found that as soon as the ionic strength of the chromatin gel was increased, precipitation occurred. For this reason it was necessary to dialyse the sucrose gradient histone transacetylase preparation to reduce its ionic strength so that the dialysed enzyme could be added to the chromatin gel (containing 500 μg total histone) and pre-incubated together to allow the histone transacetylase to find its place on chromatin before the other incubation constituents which caused immediate precipitation were added (Table 2.6.2.IV).

Table 2.6.2 shows the results obtained with the dialysed histone transacetylase preparation. The incubations using acid extracted total histones were repeated using undialysed enzyme (Table 2.6.3, I + II) and in addition isolated histone subfractions (4.5.6.1) were also incubated with the histone transacetylase (Table 2.6.3.III). 500 μg of calf

TABLE 2.6.2

SUBSTRATE SPECIFICITY OF HISTONE TRANSACETYLASE
(DIALYSED) WITH VARIOUS HISTONE PREPARATIONS

	Substrate	dpm incorporated
I	Calf thymus total histone (acid extracted) (500 µg)	3634
II	Rat liver total histone (acid extracted) (500 µg)	4138
III	Rat liver histone (protamine displaced) (500 µg)	5074
IV	Rat liver chromatin (\equiv 500 µg total histone)	No incorporation*

(* over and above the endogenous acetylation of the chromatin preparation in the absence of added enzyme). Substrates were prepared as described in 4.5.1, 4.5.3, 4.5.4 and 4.5.2.2 respectively. Incubation constituents, initiation and termination of the reaction and preparation of histones for radioactivity determination are given in 4.3.

thymus total histone was calculated to contain approximately 100 µg of histone H1, 80 µg H2A, 115 µg H2B, 120 µg H3 and 80 µg H4 which amounts were incubated with the histone transacetylase (Table 2.6.3.III), by assuming that the molar ratios of the various histone fractions are histone H1 : H2A : H2B : H3 : H4 = 0.5 : 1 : 1 : 1 : 1 (Johns, 1967; Kornberg, 1974). To study whether there is any difference in the acetylation of the subfractions when incubated separately or when recombined, these amounts of the isolated subfractions were mixed together and incubated with histone transacetylase (Table 2.6.3.IV).

TABLE 2.6.3

SUBSTRATE SPECIFICITY OF HISTONE TRANSACETYLATING
(UNDIALYSED) WITH VARIOUS HISTONE PREPARATIONS

Substrate		dpm incorporation
I	Calf thymus total histone (acid extracted) (500 µg)	2810
II	Rat liver total histone (acid extracted) (500 µg)	4030
III	Calf thymus histone	1558
	100 µg histone H1	
	subfractions (≡ 500 µg	80 µg histone H2A
	total histone)	115 µg histone H2B
		120 µg histone H3
		80 µg histone H4
		<u>1689</u>
		ε = 8445
IV	Calf thymus histone subfractions recombined (≡ 500 µg total histone)	3363

Substrates were prepared as described in 4.5.1, 4.5.3 and 4.5.6.1 respectively. Incubation constituents, initiation and termination of the reaction and preparation of the histones for radioactivity determination are given in 4.3.

2.7

DISCUSSION

An isolation procedure was developed for the rapid preparation of histone transacetylase from rat liver nuclei which could be completed in only 4 days. As mentioned before (2.1) no attempt was made to purify the histone transacetylase to homogeneity since it was used merely as a tool to study the sites of histone acetylation occurring in vitro. Further purification of the sucrose gradient fractions could be obtained by ion-exchange or gel chromatography (Table 2.2.3.1), but the resulting preparation either gave an uneconomical yield of histone transacetylase activity when the further purification of 1.6 to 3x was considered (Table 2.2.3.1) or else was unstable upon storage (Table 2.5.1). For these reasons, as well as the fact that column runs were undesirable when a rapid enzyme isolation procedure was required, further purification of the sucrose gradient fractions was not performed for routine enzyme isolations.

It was essential that the histone transacetylase preparation used be histone-free to enable substrate specificity to be studied. Lue et al. (1973) found that if sonication of nuclei was done in 0.3 M ammonium sulphate - 1 M sucrose, mainly histone associated histone transacetylase was extracted, whereas if the nuclei were extracted with a hypotonic medium, mainly histone-free histone transacetylase was solubilised. Therefore the nuclei were sonicated at a low ionic strength. An increase in the salt concentration of the sucrose gradient caused a shift of the histone transacetylase activity towards the top of the gradient (Fig. 2.2.2.1) and this was interpreted to mean that histone-associated histone transacetylase is still extracted from the nuclei or else that histone transacetylase is able to interact with solubilised histones after extraction from the nuclei. However 0.3 M NH_4Cl clearly causes a dissociation of these contaminating histones. Lue et al. (1973) also found that histone-associated histone transacetylase was not bound to DEAE-Sephadex and since it has been found (Fig. 2.2.3.1) that all of the activity is bound to DEAE-cellulose, the sucrose gradient fractions were assumed to be in the histone-free form. This was confirmed by gel electrophoresis which showed that the sucrose gradient fractions were histone-free.

Table 2.6.1 shows that the enzyme extract exhibits a marked preference

for total histone as substrate. The possibility that another better substrate for the enzyme exists is not excluded since only a few proteins have been tested as substrates. However since it was found that this preparation exhibits the same substrate site specificity towards histones as occurs in vivo (see Part 3) it is likely that this enzyme is indeed a histone transacetylase.

The addition of exogenous histone transacetylase causes no increase in the acetylation of chromatin-bound histones above that catalysed by the endogenous chromatin-bound histone transacetylase activity (Table 2.6.2). This may be due to either (a) all of the histone transacetylase binding sites on chromatin being filled by the endogenous histone transacetylase or (b) chromatin-bound histones are not the substrate for the isolated histone transacetylase or (c) the exogenous histone transacetylase cannot get access to the acetylation sites on histone due to steric hindrance. Because of this result chromatin was not used as the substrate for the study of the substrate specificity of the histone transacetylase preparation. Table 2.6.2 shows that rat histones prepared by protamine displacement exhibit the highest ^3H -acetyl incorporation. It was thus decided to use the rat liver protamine-displaced histone as substrate since next to chromatin it is the histone transacetylase substrate most closely resembling that existing in vivo as discussed earlier (2.6). Table 2.6.3 (III and IV) shows that when isolated histone subfractions are incubated with histone transacetylase the counts incorporated are very much more than when the subfractions are re-combined again. It is possible that there is competition between the histone subfractions for the binding of histone transacetylase or else when histone aggregates are formed that less acetylation sites are available. Table 2.6.3.III shows that the relative susceptibility of the histone subfractions to acetylation is $\text{H2B} > \text{H3} > \text{H4} > \text{H1} > \text{H2A}$ a result which is in contrast to that found when protamine displaced histone is acetylated by histone transacetylase (see Fig. 3.3.3.1.1) where $\text{H4} > \text{H3} > \text{H2A}, \text{H2B}, \text{H1}$. The results obtained from this experiment emphasise the different acetylation which occurs when different histone preparations are used as substrate.

This histone transacetylase preparation was used (3.2) to prepare ^3H -acetyl histone using rat liver total histone (protamine displaced) as substrate, by incubation under the optimal conditions determined above.

The specific activity and yield of ^3H -acetyl histone were sufficient to enable sequencing studies of ^3H -acetyl histones to be undertaken (see Part 3).

PART 3

INVESTIGATION OF ENZYMATICALLY ACETYLATED ^3H -ACETYL HISTONE3.1 INTRODUCTION

In Part 2 the isolation of a crude histone-free histone transacetylase preparation was presented. The substrate specificity of this histone transacetylase preparation was also investigated. Part 3 describes the preparation of ^3H -acetyl rat liver total histone (protamine displaced) by enzymatic acetylation with the histone transacetylase preparation, using the optimal incubation conditions for acetylation determined in Part 2, in sufficient quantity and with high enough specific activity to enable sequencing studies of the histone subfractions most susceptible to acetylation (histones H3 and H4) prepared from the total histone to be undertaken.

3.2 PREPARATION OF ENZYMATICALLY ACETYLATED ^3H -ACETYL RAT LIVER TOTAL HISTONE (PROTAMINE DISPLACED)

Protamine displaced rat liver histone was incubated with the histone transacetylase and ^3H -acetyl CoA and the crude ^3H -acetyl histone recovered as described in 4.5.5. 125 mg (approximately 8 μmole) of ^3H -acetyl histone containing 5×10^8 dpm (approximately 84 nmole acetate) was recovered after freeze drying. (Specific activity 4×10^6 dpm/mg protein, or approximately 0.01 mole acetate/mole histone).

3.3 FRACTIONATION OF ^3H -ACETYL TOTAL HISTONE3.3.1 Preparation of ^3H -acetyl histone H4

^3H -acetyl total histone (125 mg) prepared as described in 3.2 was subjected to gel filtration on Biogel P-60 (4.5.6.2.1) using the method of Böhm et al. (1973). Fig. 3.3.1 shows that histones H1 and H4 eluted as chromatographically pure fractions whereas histones H2A/H3 and H2B

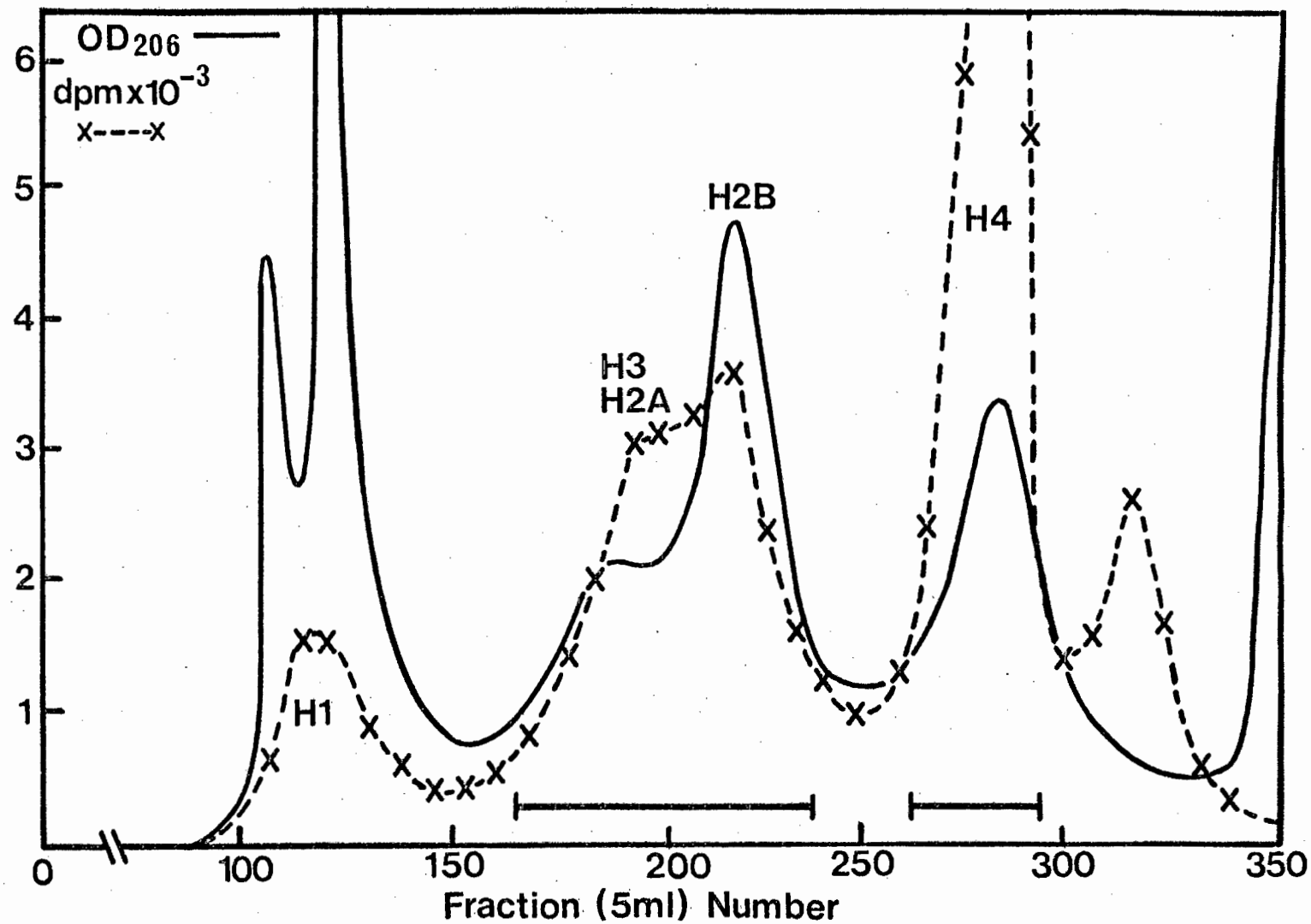


Fig. 3.3.1 : Biogel P-60 chromatography of ³H-acetyl total histone. Enzymatically acetylated ³H-acetyl rat liver total histone (protamine displaced) was prepared as described in 3.2. Chromatography was performed as described in 4.5.6.2.1 with 10 μ l aliquots being taken for radioactivity determinations.

were not resolved from one another. The small peak of radioactivity eluting after H4 but before the urea-mercaptoethanol peak has not been identified. Histone H4 contained in the pooled fractions indicated in Fig. 3.3.1 was found to be electrophoretically pure. After freeze drying the pooled fractions, the recoveries of protein were found to be histone H1 = 32 mg; histone H4 = 20 mg and histone H2A/H2B/H3 = 53 mg which represents 84% recovery of the protein applied to the column. The specific activities of the H4 and H2A/H2B/H3 fractions were found to be 3.76×10^6 dpm/mg and 1.13×10^6 dpm/mg protein respectively. Approximately 46% of the radioactivity applied was recovered from the column. Since the overall recovery of protein from the column exceeded 84%, it was concluded that a labelled contaminant had been removed by adsorption onto the gel used to pack the column. Gel electrophoresis of the total histone (see 3.3.3.1) also only gave a ^3H -recovery of about 42%.

3.3.2 Preparation of ^3H -acetyl histone H3

The histone H2A/H2B/H3 mixture (53 mg protein, 6×10^7 dpm) obtained from Biogel P-60 elution (3.3.1) was oxidised with o-iodosobenzoate and subjected to Sephadex G-100 chromatography using the method of Brandt and von Holt (1971) described in 4.5.6.2.2. Fig. 3.3.2 shows that the histone H3 dimer was eluted well before the histone H3 monomer as well as histones H2A and H2B. After the fractions containing $(\text{H3})_2$ has been pooled, dialysed and freeze dried 6.13 mg of $(\text{H3})_2$ containing 1×10^7 dpm were recovered, with a specific activity of 1.6×10^6 dpm/mg. Recovery of radioactivity from the column was 95%. If it is assumed that histones H2A/H2B and H3 are present in equal amounts by weight in the peak obtained from Biogel P-60 then the yield of 6.13 mg of histone H3 dimer is only about 35% of the expected yield.

3.3.3 Characterisation of ^3H -acetyl rat liver histones

3.3.3.1 Gel electrophoresis

Fig. 3.3.3.1.1 shows that histones H4 and H3 are the fractions which are most acetylated in the total histone prepared in 3.2. The 47 μg of enzymatically acetylated total histone subjected to gel electrophoresis contained 1.88×10^5 dpm and only 42% of this radioactivity was recovered

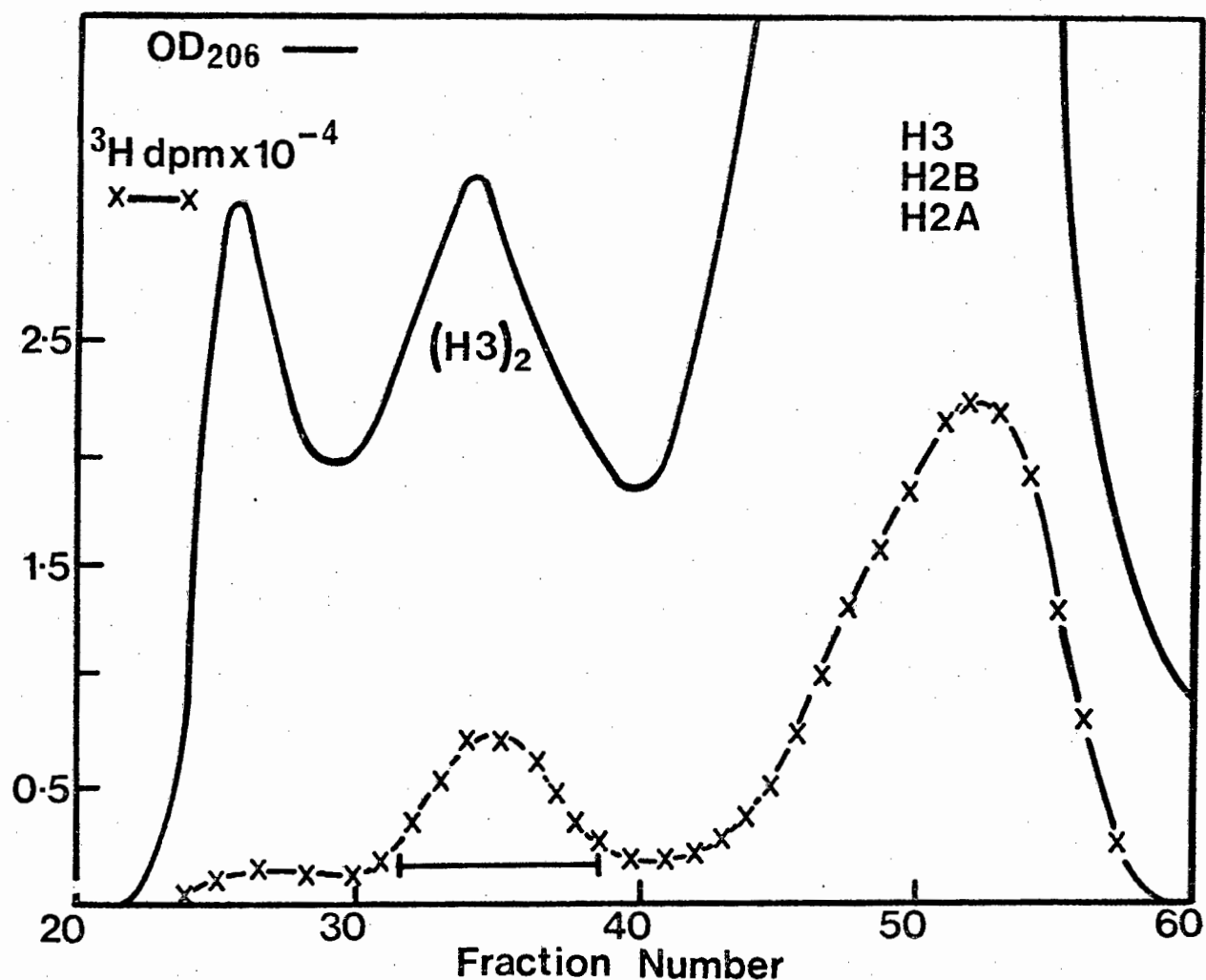


Fig. 3.3.2 : Sephadex G-100 gel filtration of iodosobenzoate oxidised H3-dimer. A 2.5 x 100 cm column equilibrated with 0.02 N HCl (pH 2.1) was packed and eluted at 80 cm head (4.5.6.2.2). Fractions of 5 ml were collected. 20 μ l aliquots were withdrawn for ^3H -counting.

on the gel, a value which agrees closely with the 46% of ^3H -recovery found after Biogel P-60 chromatography (3.3.1). Fig. 3.3.3.1.1 also shows that non-enzymatic acetylation of histone was negligible (4.7% of enzymatic acetylation) under the incubation conditions used to prepare enzymatically acetylated histone (3.2).

Histone H3 dimer was subjected to polyacrylamide gel electrophoresis in the absence or presence of β -mercaptoethanol. Fig. 3.3.3.1.2 shows that upon addition of β -mercaptoethanol, the histone H3 dimer is converted into monomer. Recovery of radioactivity in both gels was about

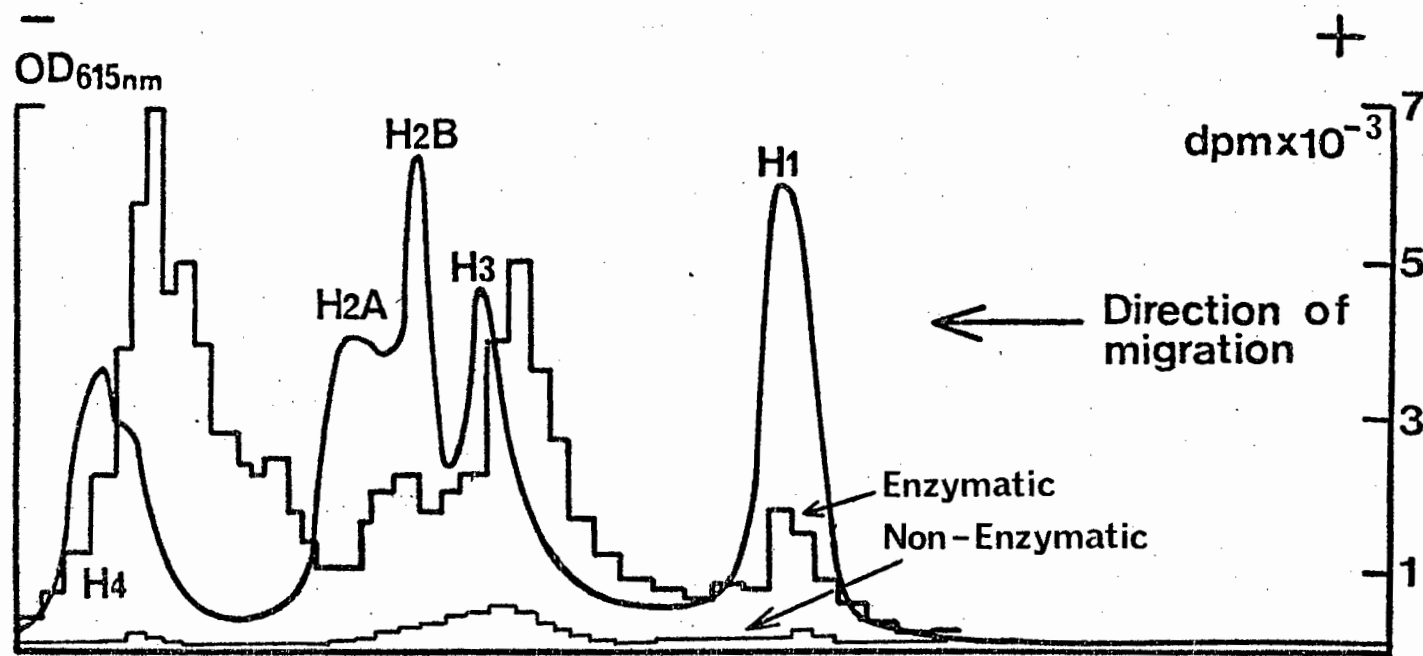


Fig. 3.3.3.1.1 : Polyacrylamide gel electrophoresis of ^3H -acetyl rat liver total histone (protamine displaced). Enzymatically or non-enzymatically acetylated total histone ($47\text{ }\mu\text{g}$) was applied to 20 cm polyacrylamide gels (4.2.5). Non-enzymatically acetylated histone was prepared by incubating rat liver total histone (protamine displaced) with ^3H AcCoA as described in 4.5.5 except that $0.3\text{ M NH}_4\text{Cl-TEM}$ instead of sucrose gradient fractions were added to the incubation. Radioactivity of the gel slices was determined after oxidation in the sample oxidiser (4.2.6).

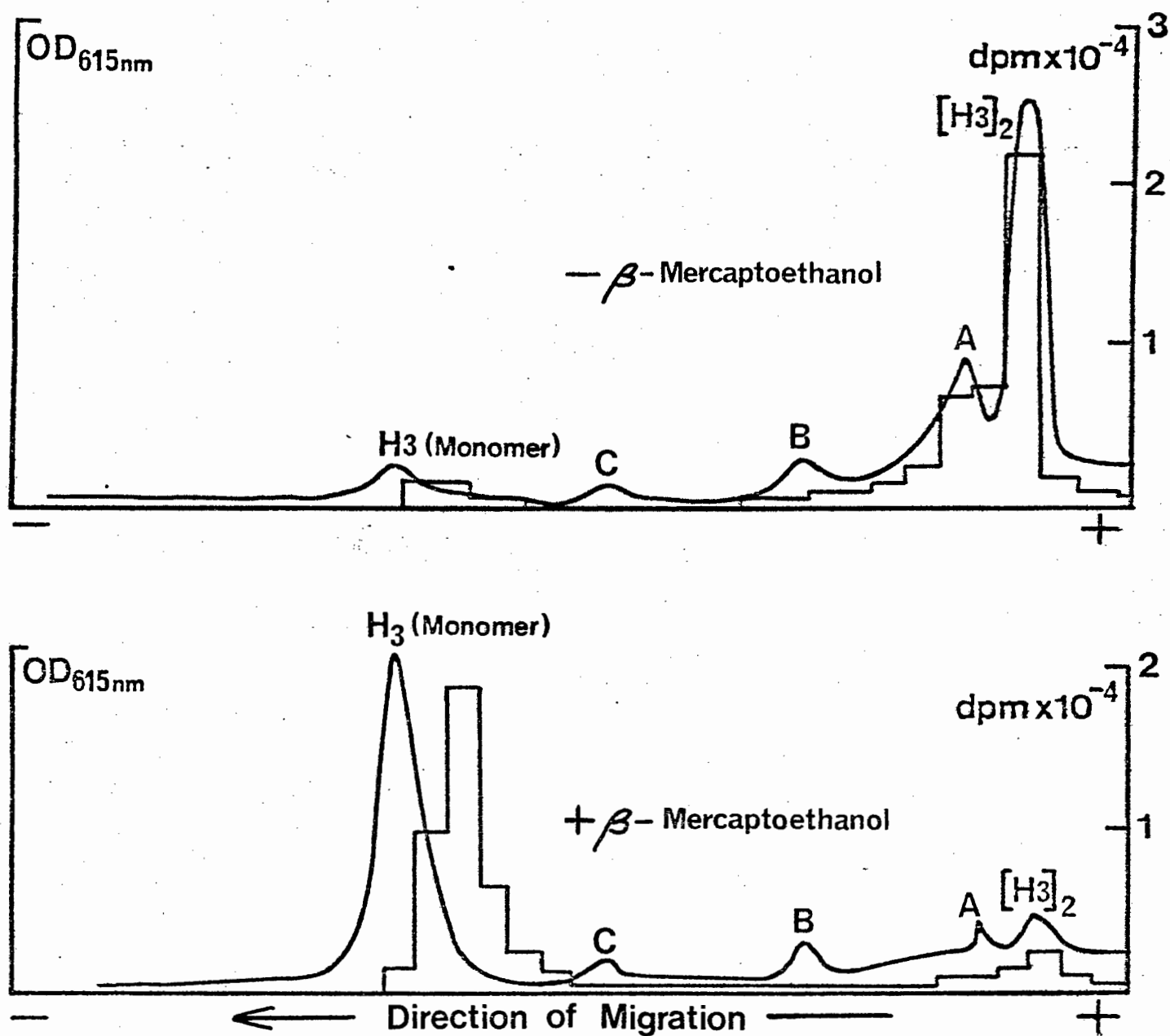


Fig. 3.3.3.1.2 : Polyacrylamide gel electrophoresis of H3 dimer in the absence or presence of reducing agent. H3 dimer (43 μ g, 69 758 dpm) prepared as described in 3.3.2 was dissolved in 8 M urea with or without β -mercaptoethanol and subjected to electrophoresis on 10 cm gels (4.2.5). Radioactivity was determined by oxidation in a sample oxidiser after slicing the gels (4.2.6).

80%. The minor electrophoretic heterogeneity of the H3 dimer (peak A, Fig. 3.3.3.1.2) may be due to the presence of histone H3 species with 1 or 2 cysteine residues which it has been suggested, occur in rodents (Garrard, 1976) or to the formation of a hybrid dimer in which only one of the partners is histone H3 or one of its degradation products (Brandt et al., 1974b). On reduction with β -mercaptoethanol the radioactivity which co-electrophoreses and co-chromatographs with the dimer appears in the monomer (Fig. 3.3.3.1.2). Other minor contaminating bands (B & C Fig. 3.3.3.1.2) are non-radioactive and do not contain any cysteine residues since their areas are the same in the absence or presence of β -mercaptoethanol.

3.3.3.2 Histone deacetylase

To confirm that the radioactivity in the ^3H -preparation indeed represents acetylated histones H3 and H4 and not a radioactive contaminant co-purified with the histone, a nuclear histone deacetylase shown to be specific for histones H3 and H4 (Vidali et al., 1972) was prepared as described in 4.6.2. Fig. 3.3.3.2.1 shows the chromatographic profile obtained on Sepharose 6B.

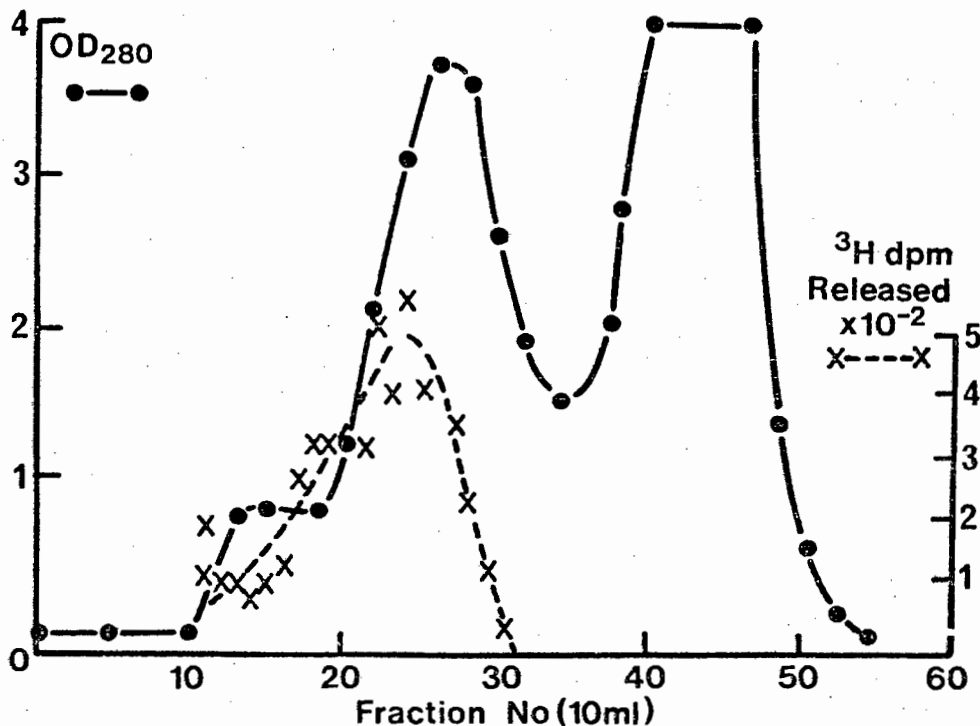


Fig. 3.3.3.2.1 ; Chromatography of histone deacetylase on Sepharose 6B. Chromatography was performed as described in 4.6.2.2. Histone deacetylase activity (x-x-) was determined using the method described in 4.6.2.3.

The histone deacetylase elutes in a position corresponding to that reported by Vidali et al. (1972). The histone deacetylase was incubated (as described in 4.6.2.3) with ^3H -histone H4 or ^3H -histone H3/H2A/H2B mixture (4.5.6.2.1). To assess the degree of deacetylation, the histone was either electrophoresed and the electrophoretic fractions determined or else the liberated ^3H -acetic acid was determined (4.6.2.3). Table 3.3.3.2.1 shows that between 13 and 19.2% of the ^3H -acetyl groups were removed from the histone. This value is fairly close to the 20% deacetylation of histone H4 reported earlier (Krieger et al., 1974) using the same enzyme. Since the histone deacetylase is specific for histones H3 and H4 (Vidali et al., 1972), and deacetylates the ^3H -preparations of histone H3 and H4 to the same extent as that reported, it can be concluded that ^3H -histone H3 and H4 are indeed the ^3H -species present in these preparations.

TABLE 3.3.3.2.1

ENZYMATIC DEACETYLATION OF HISTONE

Substrate	Deacetylase assay method	% Removal of ^3H -acetate relative to zero time incubations
H2A/H2B/H3 mixture	Electrophoresis	14%
Histone H4	"	13%
H2A/H2B/H3 mixture	Ethyl acetate extraction of ^3H acetic acid	19.2%
Histone H4	"	13.8%

Histone H2A/H2B/H3 mixture (70 μg) or histone H4 (20 μg) were incubated with deacetylase as described in 4.6.2.3. All 60 min incubations were corrected for zero time incubations. For the electrophoretic determination of the degree of deacetylation, the total dpm occurring in the single radioactive peak found on the gels was determined. For the ethyl acetate extraction method, the total dpm extracted into the ethyl acetate layer were determined.

3.4

PREPARATION OF HISTONE H4 PEPTIDES

Sequential degradation of intact histone H4 to reveal the internal ϵ -N-acetyl lysines is not possible because the N-terminal serine has a blocked, acetylated α -amino group (DeLange et al., 1969a). However these authors have described (DeLange et al., 1969a) a method utilising chymotryptic and tryptic hydrolysis for the generation and purification of histone H4 peptides. The method used is summarised diagrammatically in Fig. 3.4. The peptide nomenclature used is that of DeLange et al., 1969a.

Whereas DeLange et al. (1969a) developed this method in order to be able to determine the amino acid sequence of histone H4, the object of the present study was to investigate the sites of ^3H -acetyl group incorporation in histone H4. For this reason the purification of ^3H -acetyl peptides was monitored by determining the radioactivity of the column effluents only rather than by determining the pattern of all the generated peptides via reaction with ninhydrin as used by DeLange et al. (1969a). The ^3H -peptide fractions obtained after chymotryptic digestion and gel filtration were clearly expected to be contaminated with other non-radioactive peptides. Amino acid analysis of the chymotryptic peptide mixtures obtained confirmed that this was indeed so (Table 3.4.1.1).

Since all the reported sites of acetylation of histone H4 are in the N-terminal region of the molecule (see Table 1.2.7), only 3 radioactive species (intact histone H4, peptides C-1 and C-1a) were expected after chymotryptic digestion of histone H4, which would elute in the order of their relative molecular weights, i.e. histone H4, and then peptide C-1 followed by peptide C-1a. Since the yield of peptide C-1 was reported to be better than that of peptide C-1a (DeLange et al., 1969a), a result which was confirmed in the present work (see Fig. 3.4.1.1) it was decided to isolate the fractions containing peptide C-1, rather than C-1a.

Since ^3H -acetylated peptide C-1 consists of amino acid residues 1-37 (see Fig. 3.4) the N-terminus is still blocked and Edman degradation still cannot be done. Tryptic digestion of the peptide after maleylation was utilised to prepare two relatively large fragments, T(M)-2 and T(M)-5 (see Fig. 3.4) as well as a number of smaller peptides, of which only

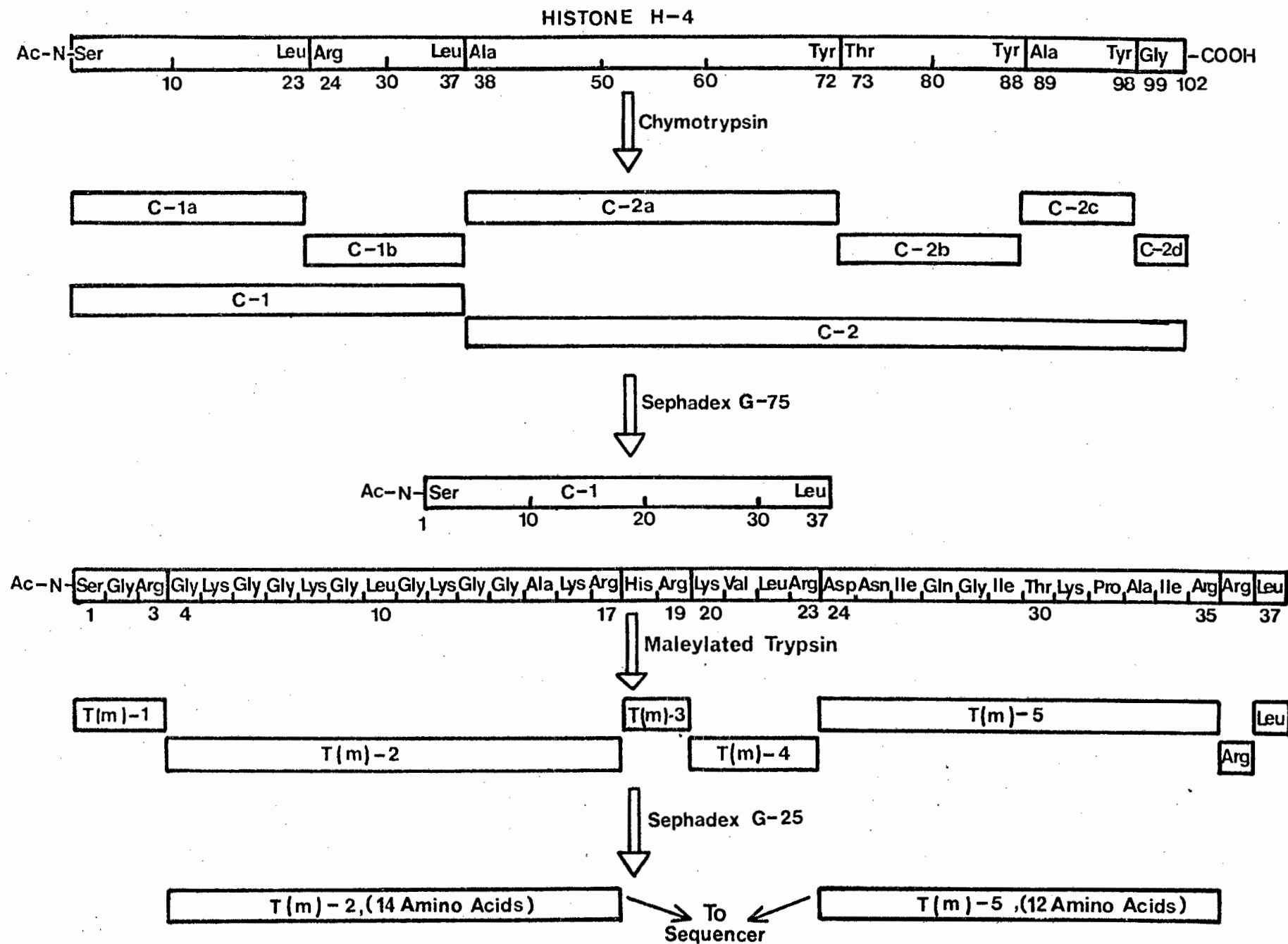


Fig. 3.4 : Generation of histone H4 peptides (DeLange et al., 1969a).

T(M)-4 contained a lysine residue (see Fig. 3.4). Gel filtration of the tryptic digest should separate T(M)-2 and T(M)-5 from the other peptides and clearly show whether T(M)-4 is acetylated or not. The sequences of T(M)-2 and T(M)-5 for calf thymus histone H4 are :

T(M)-2 : Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-Lys-Arg

T(M)-5 : Asp-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg-Arg-Leu

(DeLange et al., 1969a). Since none of the 4 lysine residues of T(M)-2 coincides in its position with the lysine residue of T(M)-5 it was possible to simultaneously sequence these 2 peptides and unambiguously assign the sites of acetylation (Fig. 3.5.2.1.1).

3.4.1 Preparation of chymotryptic ^3H -histone H4 peptides

Chymotryptic hydrolysis was performed using the method of DeLange et al. (1969a) described in 4.7.1. The NaOH consumption indicated that 2.85 peptide bonds/histone H4 molecule had been cleaved. This was less than the 6 bonds cleaved per molecule reported earlier (DeLange et al., 1969a).

It was found in a pilot run that when the chymotryptic hydrolysate was applied to Sephadex G-25 under the conditions used by DeLange et al. (1969a) that only a single ninhydrin positive peak was obtained, eluting at the outer volume of the column, in contrast to the 3 unresolved peaks reported by DeLange and co-workers. For this reason Sephadex G-75 was used for the chromatographic purification of chymotryptic histone H4 peptides. Fig. 3.4.1.1 shows that 3 peaks of radioactivity were obtained which were postulated to be : peak I - intact ^3H -histone H4; peak II - ^3H -peptide C-1; peak III - ^3H -peptide C-1a (see Fig. 3.4 for histone H4 peptide nomenclature).

This assignment was made because (a) it is assumed that the peptides would elute in the order of their relative molecular weights, i.e. H4 histone first followed by C-2, C-1, C-2a, C-1a, C-2b and C-1b, 2c and 2d (see Fig. 3.4); (b) it was also assumed that acetylation most probably only occurred in the N-terminal region of the molecule as reported earlier (Table 1.2.7); (c) amino acid analysis (4.7.3) of peaks I-III obtained after chymotryptic hydrolysis and Sephadex G-75 chromatography

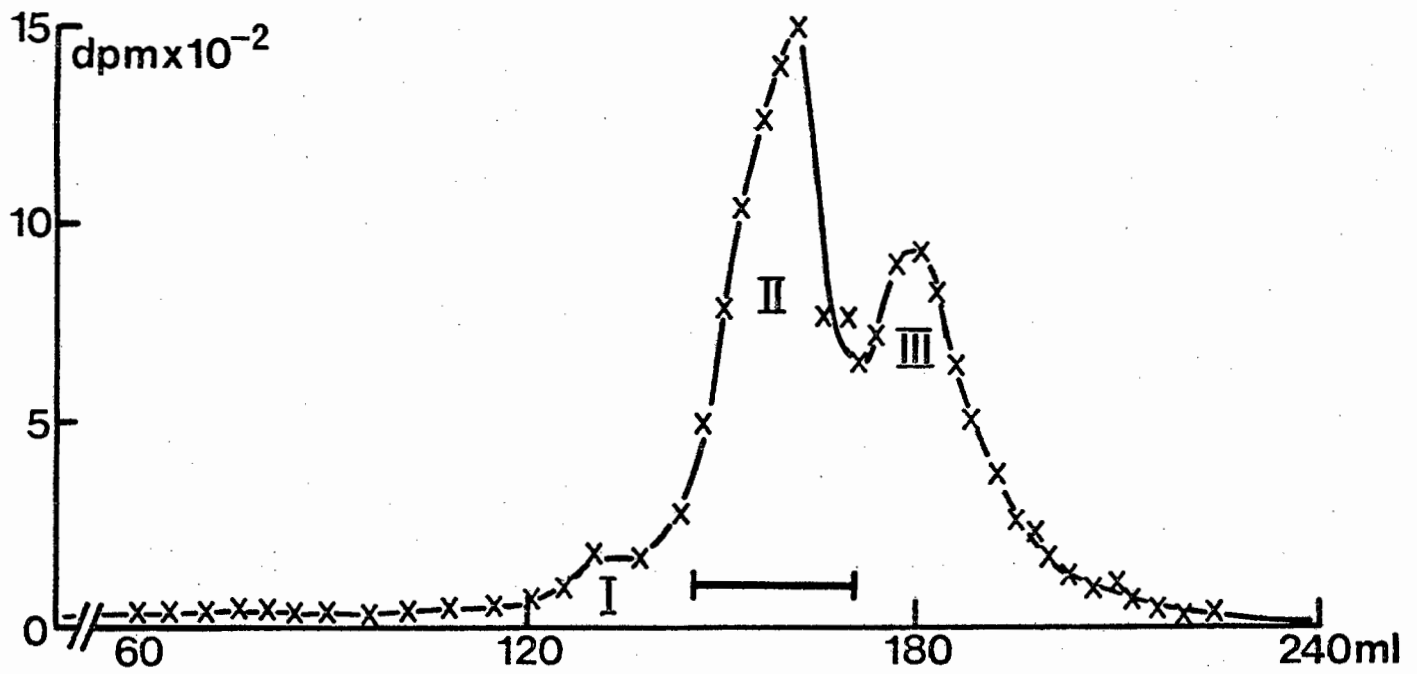


Fig. 3.4.1.1 : Gel filtration of chymotryptic histone H4 hydrolysis products. Chymotryptic hydrolysis was performed as described in 4.7.1 and the Sephadex G-75 column was eluted as described in 4.7.2.

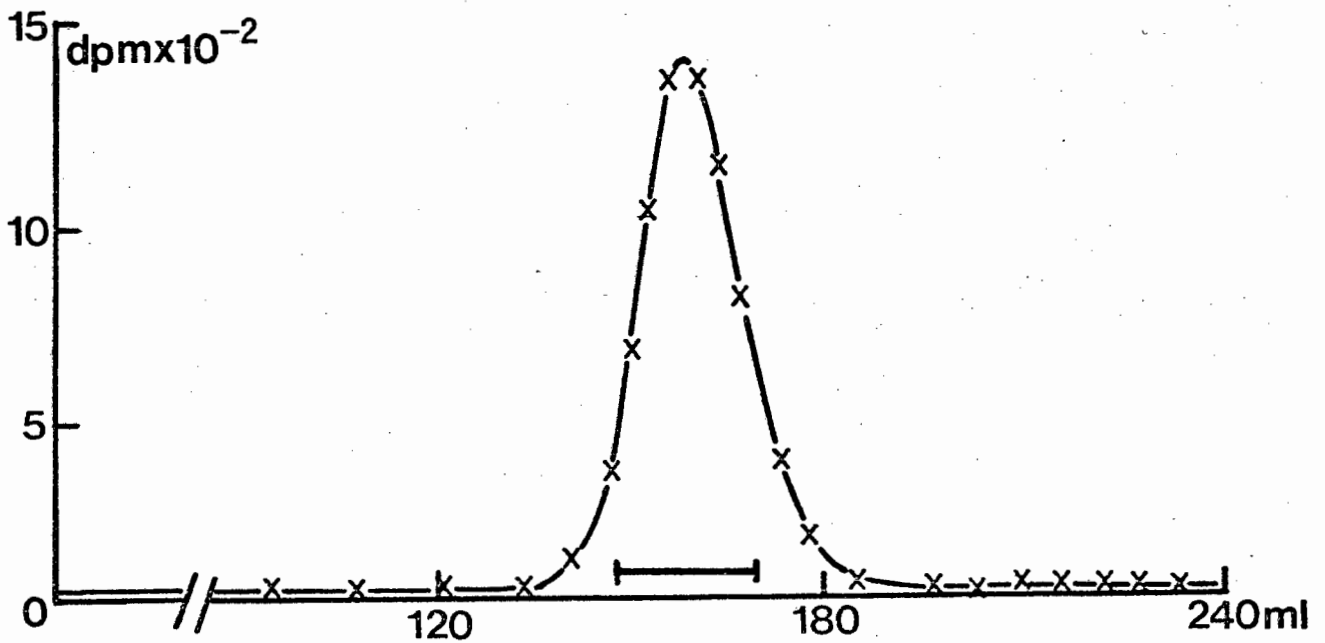


Fig. 3.4.1.2 : Gel filtration on Sephadex G-75 of peak II from Fig. 3.4.1.1 reappplied to the column.

in a pilot run (Table 3.4.1.1) supported this assignment of ^3H -peptides to explain the radioactive peaks obtained on Sephadex G-75 elution although as expected there is extensive contamination by other peptides present. The amino acid composition of these fractions is intermediate between the expected analysis for the pure peptide fraction and other peptides which could be expected to be contaminating these fractions (Table 3.4.1.1); (d) if peptides C-2 and C-2a were radioactive, the sharp peaks obtained (Fig. 3.4.1.1) would be expected to broaden out considerably.

Fraction II (Fig. 3.4.1.1) on rechromatography (Fig. 3.4.1.2) appeared to be homogeneous and was assumed to be ^3H -C-1 peptide with other (non-radioactive) peptides as contaminants of the ^3H -peptide (Table 3.4.1.1).

The recovery of radioactivity from these two Sephadex G-75 steps was 76.5% and 99.2% respectively. Peak II obtained from the run shown in Fig. 3.4.1.1 contained 2.7×10^6 dpm and after rechromatography 2.1×10^6 dpm was recovered in the 2.1 mg of peptide obtained.

3.4.2 Preparation of tryptic ^3H -histone H4 peptides

Peptide C-1 obtained as described in 3.4.1 was maleylated and subsequently digested with trypsin as described in 4.7.4 and 4.7.5. After Sephadex G-25 chromatography (Fig. 3.4.2.1) a single radioactive fraction probably containing the 2 peptides T(M)-2 and T(M)-5 of approximately equal size (refer to Fig. 3.4), and 84% (i.e. 1.03×10^6 dpm, 0.75 mg peptide) of the radioactivity recovered from the column resulted. The simultaneously generated smaller peptides T(M)-1 and T(M)-3 do not contain lysine which could undergo acetylation, only the small peptide T(M)-4 contains lysine which however has been reported to undergo methylation in vivo (see Table 1.2.2.2). Fig. 3.4.2.1 does not give any indication of the presence of a small radioactive peptide resembling T(M)-4. Aliquots of the unresolved T(M)-2 and T(M)-5 mixture were used for automatic Edman degradation (see 3.5.2).

3.4.3 Paper chromatography of tryptic histone H4 peptides

Aliquots of the radioactive peak obtained from Sephadex G-25 supposed to

TABLE 3.4.1.1

AMINO ACID ANALYSIS OF CHYMOTRYPTIC PEPTIDE MIXTURES

Amino acid	Number of residues								
	Frag- ment: Histone H4	Peak I Histone H4	C-2	C-1	Peak II (C-1)	C-2a	C-1a	Peak III (C-1a)	C-2b
Lys	11	7	5	6	6	2	5	5	2
His	2	1	1	1	1	0	1	1	1
Arg	14	10	8	6	6	5	3	4.5	1
Trp	-	-	-	-	-	-	-	-	-
Asp	5	4.6	3	2	2	2	0	2	1
Thr	7	6	6	1	3	2	0	1.6	3
Ser	2	2.6	1	1	1	1	1	1.4	0
Glu	6	7	5	1	3	3	0	2.4	1
Pro	1	0.6	0	1	0.4	0	0	0	0
Gly	17	9	8	9	4	4	8	8	0
Ala	7	7	5	2	3	2	1	4	2
CySH	-	-	-	-	-	-	-	-	-
Val	9	9	8	1	4	5	1	2.4	3
Met	1	2	1	0	1	0	0	0.5	1
Ile	6	5	3	3	2	3	0	3	0
Leu	8	6	5	3	3	3	2	3	0
Tyr	4	4	4	0	1.7	2	0	0.7	1
Phe	2	2	2	0	0.8	1	0	0.4	0
Total	102	82	65	37	42	35	22	40	16

Amino acid analysis of material contained in peaks I to III (Fig. 3.4.1.1) was done as described in 4.7.3. The amino acid compositions of intact histone H4 and the chymotryptic peptides are calculated from the amino acid sequence of histone H4 reported by DeLange et al. (1969a).

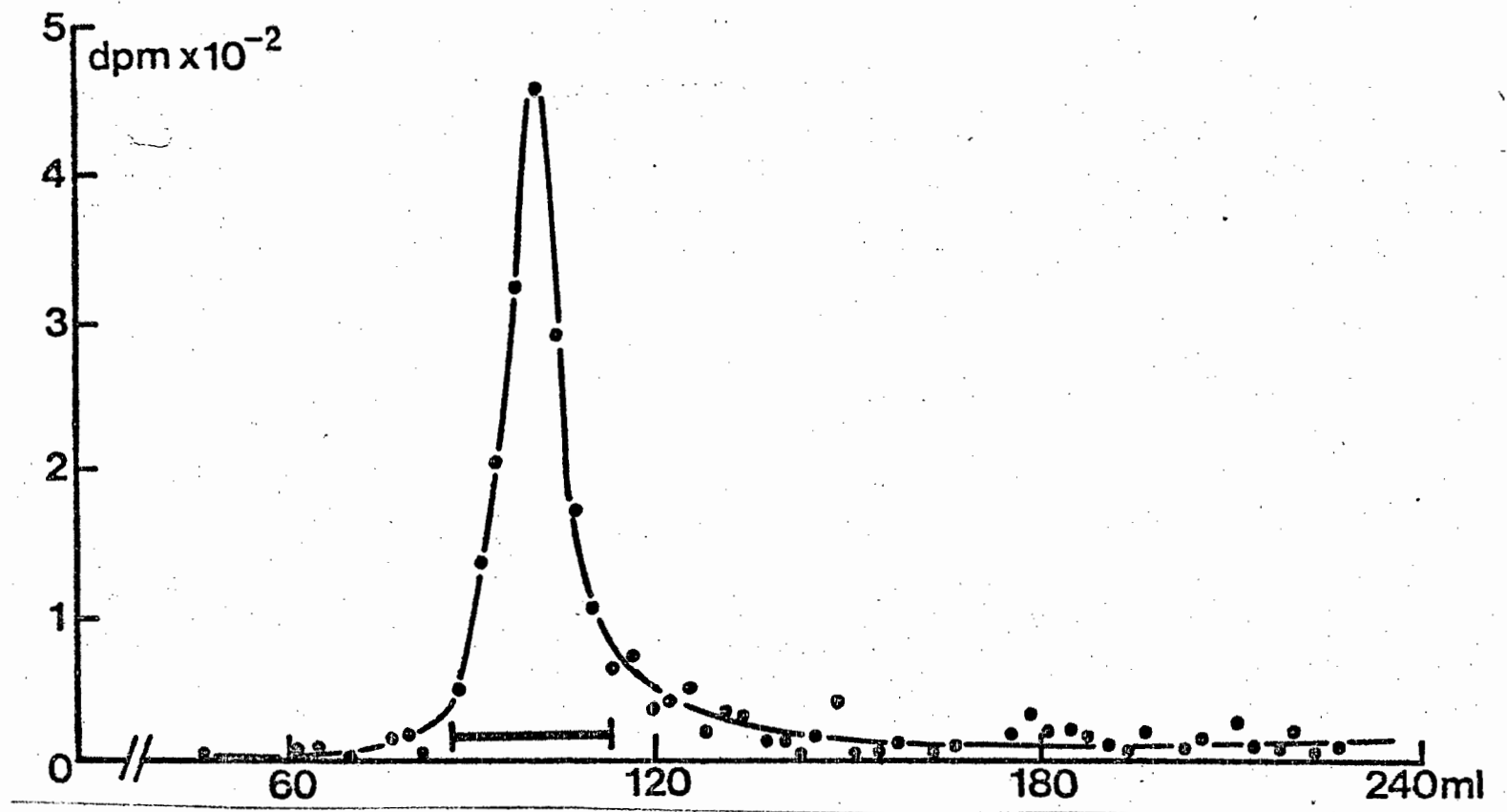


Fig. 3.4.2.1 : Sephadex G-25 chromatography of tryptic hydrolysate of C-1 peptide.
Chromatography was performed as described in 4.7.6.1.

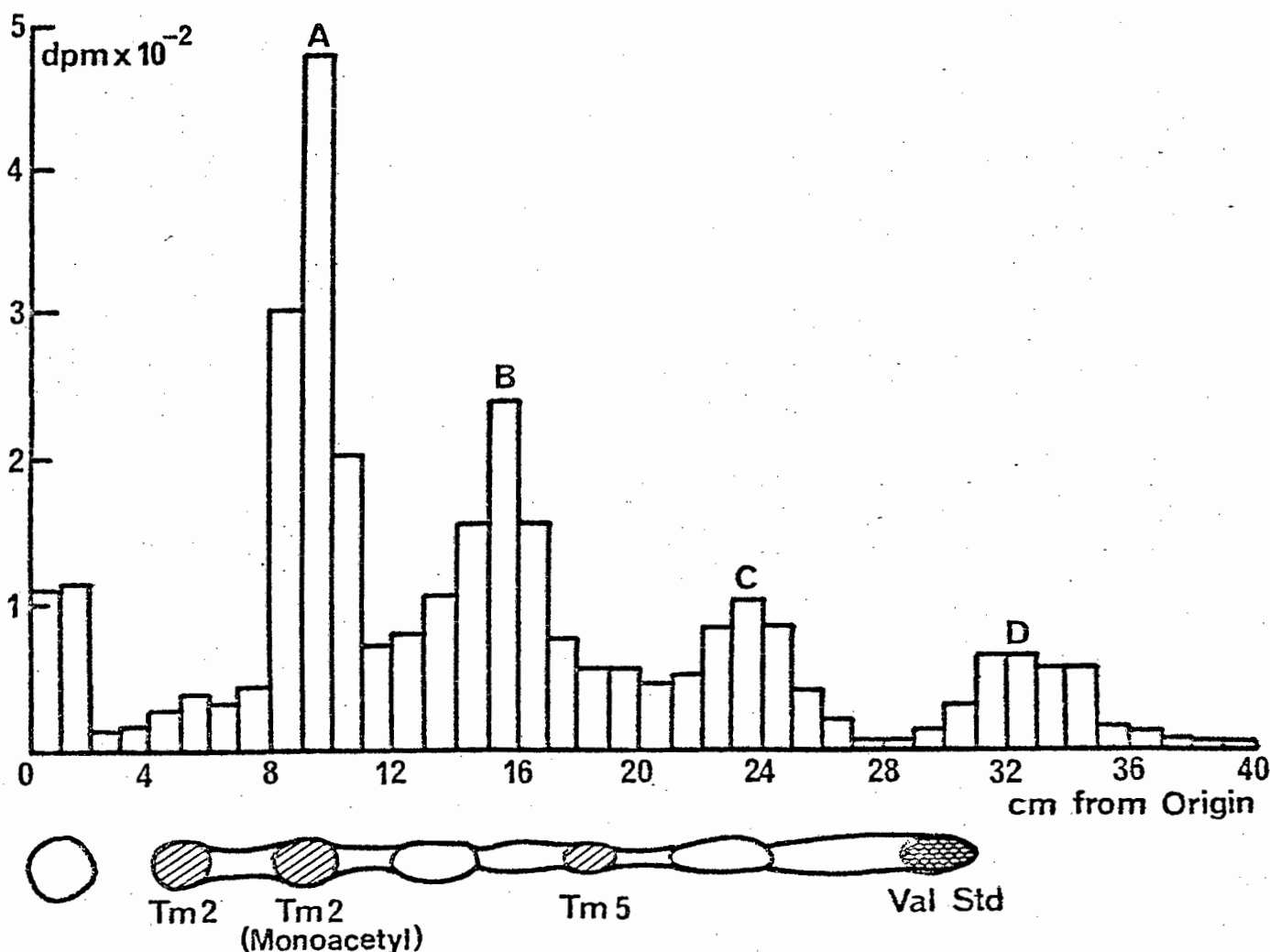


Fig. 3.4.3.1 : Paper chromatography of peptides T(M)-2 and T(M)-5. 300 μ g of peptides T(M)-2 and T(M)-5 mixture prepared by Sephadex G-25 chromatography (3.4.2) containing 4 500 dpm were subjected to paper chromatography as described in 4.7.6.2. Radioactivity was determined by oxidation of 1 cm strips of the chromatogram after visualisation of the peptides by ninhydrin spraying (4.7.6.2).

contain T(M)-2 and T(M)-5 (3.4.2) were subjected to paper chromatography as described in 4.7.6.2. Fig. 3.4.3.1 shows the radioactivity profile and ninhydrin positive material which was obtained in a pilot run. The mixture is resolved (Fig. 3.4.3.1) into four radioactive fractions differing from each other in R_f -values by equal increments. Fraction A has been shown by DeLange et al. (1969a) to be mono-acetylated T(M)-2; fractions B, C and D probably represent T(M)-2 species with 2-, 3- and 4 acetyl residues. In a preparative run 0.19 mg (approximately 60 nmole

of each of T(M)-2 and T(M)-5 of peptide mixture containing 2.5×10^5 dpm was applied to the paper. After elution of the peptides from the paper (4.7.6.2), 65% of the counts applied were recovered. Peak A (mono-acetylated T(M)-2) contained 3.8×10^4 dpm and half of this material was subjected to automatic Edman degradation (see 3.5.2.2).

3.5 AUTOMATIC EDMAN DEGRADATION

3.5.1 Sequencing of histone H3

^3H -histone H3 dimer (3.3.2) was subjected to Edman degradation as described in 4.8.1. A pilot run showed that after 30 degradation cycles 83% of the radioactivity applied was found in the ethyl acetate layer after conversion to the PTH-amino acid (4.8.2) while 11% was found in the aqueous layer with only 6% of the counts applied being left in the sequencer cup. This indicates that all of the radioactivity occurs in the N-terminal region (residues 1-30) of the histone H3 molecule. It was also found that when aliquots of radioactive PTH-amino acids were subjected to thin layer chromatography (Fig. 3.5.1.1) that the radioactivity co-migrated with authentic ϵ -N-acetyllysine. Though the pilot run indicated higher radioactivity in lysine positions, the background radioactivity in other positions was high indicating either extractive losses of the small amounts of histone H3 applied to the cup or a considerable degree of incomplete cleavage with the resulting overlap.

When another sample of ^3H -histone H3 (2 mg, 130 nmole, 3.2×10^6 dpm) was subjected to Edman degradation, unlabelled histone H3 (1 mg, 65 nmole) was co-sequenced as carrier. A repetitive yield of 91% for each cycle of degradation was calculated from the yields of alanine in cycles 1, 7 and 15 (Table 3.5.1.1). Table 3.5.1.1 also shows that the neutral PTH-amino acid residues determined by gas chromatography were those expected from the known sequence of calf thymus histone H3 (DeLange et al., 1972).

The distribution of radioactivity obtained when the ^3H -PTH amino acid in the ethyl acetate layer was determined and corrected for 91% repetitive yield is shown in Fig. 3.5.1.2. The repetitive yield of 91% during degradation caused a gradually increasing appearance of ϵ -N- ^3H -acetyllysine-PTH at each cycle. This background radioactivity amounted at residue 3 and 5 to 0.112×10^5 and 0.31×10^5 dpm to increase at residue 22 and 24 to 2.64×10^5 and 2.63×10^5 dpm respectively. The proline

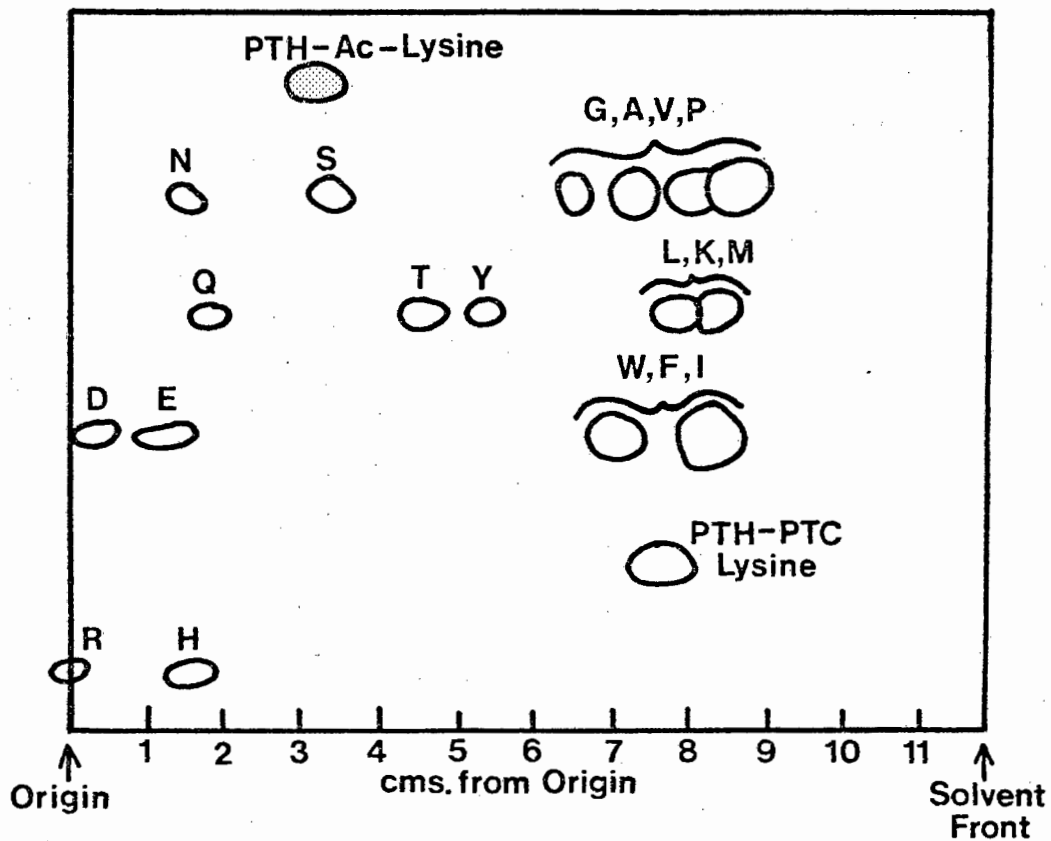
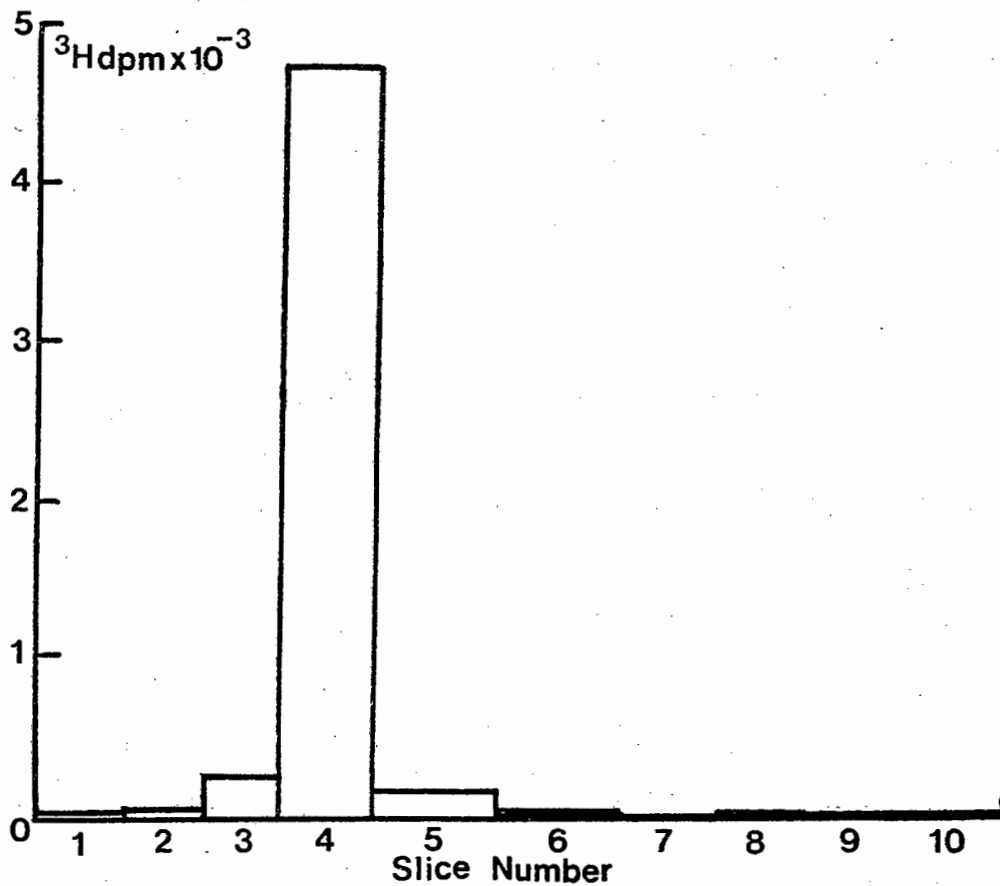


Fig. 3.5.1.1 : Thin layer chromatography of PTH-amino acids.

Thin layer chromatography was done as described in 4.8.3. Radioactivity was determined by scraping off the plates and counting the material removed (4.8.3). The radioactivity profile of residue 14 after Edman degradation (4.8.1) is shown. Single letter notation for amino acids is used.

TABLE 3.5.1.1

SEQUENTIAL DEGRADATION OF HISTONE H3
YIELDS OF SOME PTH-DERIVATIVES

Step no. (n)	Prominent amino acid				Most likely residue	Expec- ted residue
	Residue	Yield in nmole Step n-1	Step n	Step n+1		
1	Ala	-	47.6	3.5	Ala	Ala
2					-	Arg
3	Thr	2.4	23.3	6.5	Thr	Thr
4					-	Lys
5					-	Gln
6	Thr	2.9	16.3	3.2	Thr	Thr
7	Ala	4.5	32.5	6.0	Ala	Ala
8					-	Arg
9					-	Lys
10					-	Ser
11					-	Thr
12	Gly	8.6	13.0	14.6	Gly	Gly
13	Gly	13.0	14.6	5.0	Gly	Gly
14					-	Lys
15	Ala	4.8	15.8	7.0	Ala	Ala
16					-	Pro
17					-	Arg
18					-	Lys
19					-	Gln
20	Leu	5.3	9.8	7.0	Leu	Leu
21	Ala	5.7	10.7	8.7	Ala	Ala
22					-	Thr
23					-	Lys
24	Ala	6.4	9.5	11.1	Ala	Ala
25	Ala	9.5	11.1	7.8	Ala	Ala
26					-	Arg
27					-	Lys
28					-	Ser
29	Ala	5.1	7.4	5.3	Ala	Ala
30					-	Pro

3 mg of histone H3 (195 nmole, 3.2×10^6 dpm) were subjected to automatic Edman degradation as described in 4.8.1. Yields of amino acids were determined from the heights of the peaks obtained from the gas chromatograph and semi-quantitated by using correction factors for standard amounts of amino acids. Basic and acid amino acids were not determined.

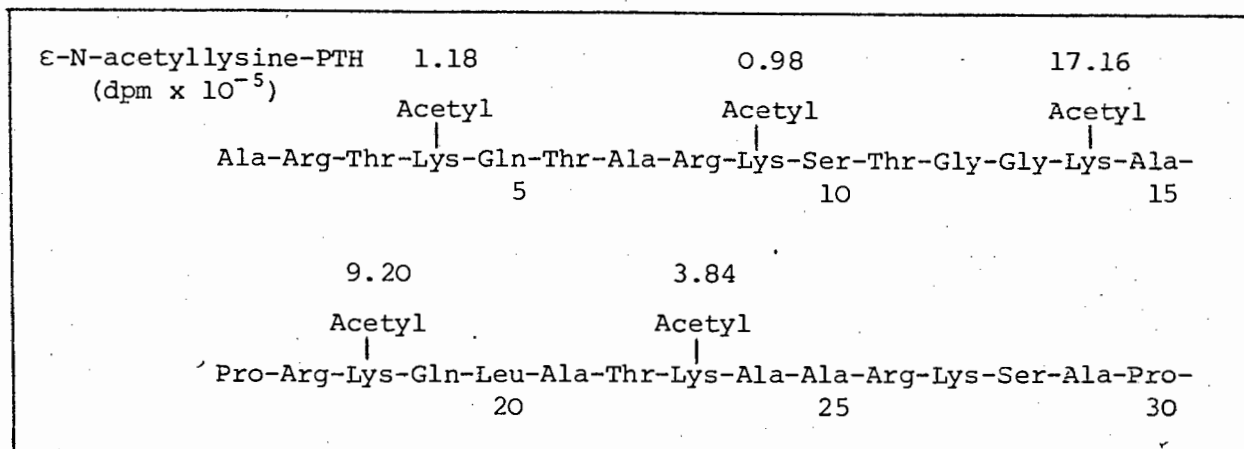


Fig. 3.5.1.2 : ^3H -acetate distribution in histone H3.

3 mg of histone H3 (3.2×10^6 dpm) was subjected to Edman degradation (4.8.1) and after conversion to the PTH-amino acid the radioactivity of the ethyl acetate layer was determined (4.8.2). Acetylation was considered significant if the radioactivity was at least twice that in the preceding degradation cycle or if the radioactivity returned after the lysine residue to the value preceding the lysine residue.

residue at position 16 which is incompletely cleaved (Brandt et al., 1977) also contributes to the background radioactivity being relatively high after residue 16.

The radioactivity increases where there is a lysine residue (positions 4, 9, 14, 18 and 23) (Fig. 3.5.1.2) but an increase was also found 2 cycles earlier than expected, i.e. at residues 2, 7, 12, 16 and 21, pointing to the possibility that the histone H3 is contaminated with a histone H3 degradation product which has the first 2 amino acids removed through proteolysis during the isolation procedure.

3.5.2 Sequencing of histone H4

3.5.2.1 Sequencing of histone H4 peptide T(M)-2 and T(M)-5 mixture

^3H -histone H4 peptide T(M)-2 and T(M)-5 mixture (0.09 mg, 1.15×10^5 dpm, containing 28 nmole of each peptide) isolated as described above (3.4.2) was subjected to automatic Edman degradation as described in 4.8.1. Pre-coupling of the peptide mixture with sulphonated-PITC and co-sequencing with unlabelled histone H4 from calf thymus (4.8.1) were necessary to

TABLE 3.5.2.1.1

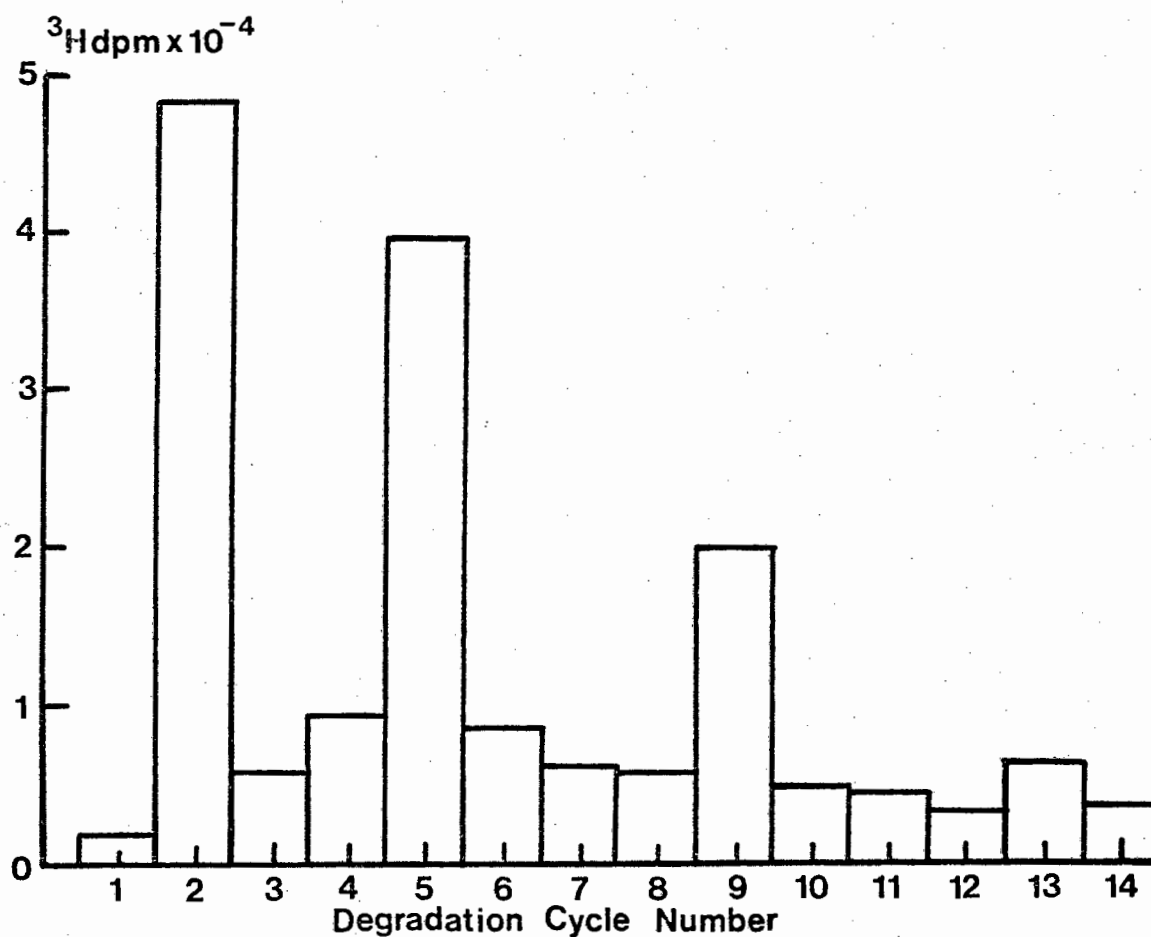
SEQUENTIAL DEGRADATION OF HISTONE H4 PEPTIDE

T(M)-2 AND T(M)-5 MIXTURE

YIELDS OF SOME PTH-DERIVATIVES

Step no. (n)	Prominent amino acid Yield in nmole				Most likely residue(s)		Expected residue(s)	
	Resi- due	Step n-1	Step n	Step n+1	T(M)-2	T(M)-5	T(M)-2	T(M)-5
1	Gly	-	6.5	5.9	Gly	-	Gly	Asp
2	-	-	-	-	-	-	Lys	Asn
3	Gly	5.9	18.3	12.4	Gly	Ile	Gly	Ile
	Ile/Leu	5.4	14.2	6.0				
4	-	-	-	-	-	-	Gly	Gln
5	-	-	-	-	-	-	Lys	Gly
6	Gly	8.7	9.6	4.8	Gly	Ile	Gly	Ile
	Ile/Leu	3.8	6.0	8.0				
7	Ile/Leu	6.0	8.0	3.4	Leu	-	Leu	Thr
8	Gly	4.8	10.4	6.2	Gly	-	Gly	Lys
9	-	-	-	-	-	-	Lys	Pro
10	Gly	6.2	9.0	9.9	Gly	Ala	Gly	Ala
	Ala	2.8	3.5	3.0				
11	Gly	9.0	9.9	6.2	Gly	Ile	Gly	Ile
	Ile	3.1	4.1	3.6				
12	Ala	3.0	4.8	3.3	Ala	-	Ala	Arg
13	-	-	-	-	-	-	Lys	Arg
14	Ile/Leu	3.6	4.1	-	-	Leu	Arg	Leu

0.09 mg of peptide mixture (28 nmole of each peptide, 1.15×10^5 dpm) was subjected to automatic Edman degradation as described in 4.8.1. Yields of amino acids were determined from the heights of the peaks obtained from the gas chromatograph and semi-quantitated by using correction factors for standard amounts of amino acids. Basic and acidic amino acids were not determined.



ϵ -N-acetyllysine-PTH (dpm $\times 10^{-4}$)	4.85	3.98	2.02
	Acetyl	Acetyl	Acetyl
Ac-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-	5	10	15
0.68			
Acetyl			
Lys-Arg-His-Arg-Lys-Val-Leu-Arg-Asp-Asn-Ile-Gln-Gly-Ile-Thr-	20	25	30
Lys-Pro-Ala-Ile-Arg-Arg-	35		

Fig. 3.5.2.1.1 : ^3H -acetate distribution in histone H4 peptide T(M)-2 and T(M)-5 mixture. Peptide T(M)-2 and T(M)-5 mixture (0.09 mg containing 28 nmole of each peptide, 1.15×10^5 dpm) prepared as described in 3.4.2 was subjected to automatic Edman degradation (4.8.1) and radioactivity of the PTH-amino acids determined (4.8.2).

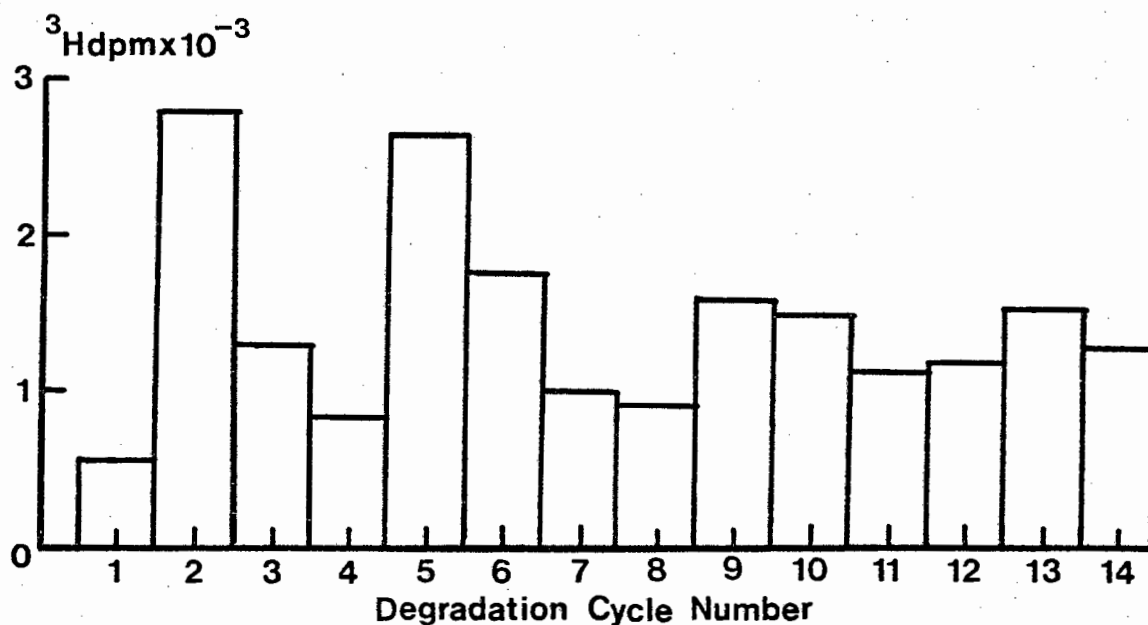
prevent all of the peptide from being washed out of the sequencer cup during the first two or three degradation cycles. The radioactivity applied was quantitatively recovered. A repetitive yield of 91% (same as for histone H3 sequencing) was assumed as it was not possible to calculate the repetitive yield from the sequence data because of the nature of the residues in the peptide mixture (Table 3.5.2.1.1). The radioactivity in the ethyl acetate layer (4.8.2) is presented in Fig. 3.5.2.1.1. It is obvious that (a) there is a gradient of acetylation which is highest near to the N-terminus of the molecule and decreases as the C-terminus is approached, and (b) that T(M)-5 is not acetylated (Lys residue 8 has only background activity). In order to determine whether the 4 acetylation sites in T(M)-2 are sequentially acetylated or whether the histone transacetylase(s) randomly acetylates any of the 4 sites, monoacetylated T(M)-2 prepared as described in 3.4.3 was also subjected to automatic Edman degradation (3.5.2.2).

3.5.2.2 Sequencing of monoacetylated T(M)-2 peptide

Monoacetylated T(M)-2 peptide prepared by paper chromatography (3.4.3) was subjected to automatic Edman degradation. Fig. 3.5.2.2.1 shows that none of the 4 acetylation sites is preferentially acetylated to the exclusion of the others.

3.6 DISCUSSION

The enzymatically acetylated total histone was found to contain 10 mmole acetate/mole total histone (3.2) before Biogel P-60 chromatography. After Biogel P-60 treatment more than 84% of the protein but only 46% of the radioactivity applied was recovered (3.3.1). Hence the degree of acetylation of the total histone is more likely to be about 5 mmole acetate/mole total histone. The ³H-histone H3 and H4 preparations isolated contained 5 and 10 mmole acetate/mole protein respectively (3.3.1 and 3.3.2). The degree of acetylation catalysed by the histone transacetylase extract isolated in Part 2 is approximately the same as that which can be calculated from the reports of acetylation catalysed by other histone transacetylase preparations. It has been reported that total histone contains between 1 and 20 mmole acetate/mole total histone after acetylation by various histone transacetylase preparations



ε-N-acetyllysine-PTH	2.82	2.68	1.61
(dpm × 10 ⁻³)			
	Acetyl	Acetyl	Acetyl
	Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-Lys-Arg		
Position in peptide		5	10
Position in protein	(5)	(10)	(15)

Fig. 3.5.2.2.1 : ³H-acetate distribution in monoacetylated T(M)-2 peptide. 1.9×10^4 dpm were subjected to automatic Edman degradation.

(Nohara, 1968; Libby, 1968; Gallwitz, 1970; Gallwitz and Sures, 1972; Horiuchi and Fujimoto, 1972; Lue et al., 1973; Candido, 1975). Reports of the level of acetylation of histone H3 were calculated at between 1 and 15 mmole acetate/mole histone H3 (Nohara, 1968; Bondy et al., 1970; Gallwitz, 1971; Racey and Byvoet, 1972; Lue et al., 1973; Candido, 1975). Histone H4 is reported to be acetylated at a level of between 1 and 7 mmole acetate/mole protein (Racey and Byvoet, 1971; Harvey and Libby, 1976). Possible reasons for these relatively low levels of acetylation could be that either (a) the histone substrate is already acetylated in vivo before exposure to the histone transacetylase and thus

there are few sites available for acetylation or (b) the majority of the isolated histone transacetylase molecules cannot bind to the correct sites once they have been dissociated.

The fractionation on Biogel P-60 (Fig. 3.3.1) of rat liver total histone did not resolve the histone H2B fraction as a separate peak, which is in contrast to the results we have reported earlier for calf thymus total histone (Böhm et al., 1973). It is probable that contaminating non-histone proteins which are present in the crude total histone preparations interfere with the separation or that the degree of modification of the histones which is different in the active liver tissue affects the aggregation pattern of the histone fractions.

Histones H3 and H4 were found to be the best acceptors of acetyl groups from acetyl CoA (Figs. 3.3.1 and 3.3.3.1.1), a result which is in agreement with previous reports (Allfrey et al., 1964; Pogo et al., 1966; Nohara et al., 1966, 1968; Vidali et al., 1968; Wilhelm and McCarty, 1970; Horiuchi and Fujimoto, 1972; Johnson et al., 1973; Candido, 1975).

The identity of ^3H -histones H3 and H4 was deduced from (a) their elution characteristics on Biogel P-60 (Fig. 3.3.1); (b) polyacrylamide gel electrophoresis of either total histone (Fig. 3.3.3.1.1) or of histone H3 dimer in the presence or absence of β -mercaptoethanol; (c) their susceptibility to the histone H3/H4 specific histone deacetylase (3.3.3.2). The identity of histone H4 was also confirmed by the peptides resulting from specific enzymatic cleavages (3.4).

The sites of acetylation of histone H3 found by Edman degradation (Fig. 3.5.1.2) are shown in a tabular form in Fig. 3.6.1.

ϵ -N-acetyllysine-PTH	1.18	0.98	17.16
(dpm $\times 10^{-5}$)	Acetyl	Acetyl	Acetyl
	Ala-Arg-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser-Thr-Gly-Gly-Lys-Ala-		
	5	10	15
	9.20	3.84	
	Acetyl	Acetyl	
	Pro-Arg-Lys-Gln-Leu-Ala-Thr-Lys-Ala-Ala-Arg-Lys-Ser-Ala-Pro-		
	20	25	30

Fig. 3.6.1 : Sites of in vitro acetylation of lysine residues in histone H3. (Data obtained from Fig. 3.5.1.2).

The sites of acetylation of histone H4 obtained from the co-sequencing of peptides T(M)-2 and T(M)-5 is shown in tabular form in Fig. 3.6.2, while the sites determined by sequencing of monoacetylated T(M)-2 are shown in Fig. 3.6.3.

ϵ -N-acetyllysine-PTH (dpm $\times 10^{-4}$)	4.85	3.98	2.02
	Acetyl	Acetyl	Acetyl
Ac-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-	5	10	15
0.68			
Acetyl			
Lys-Arg-His-Arg-Lys-Val-Leu-Arg-Asp-Asn-Ile-Gln-Gly-Ile-Thr-	20	25	30
Lys-Pro-Ala-Ile-Arg-Arg-	35		

Fig. 3.6.2 : Sites of in vitro acetylation of lysine residues in histone H4. (Data obtained from Fig. 3.5.2.1.1).

ϵ -N-acetyllysine-PTH (dpm $\times 10^{-3}$)	2.82	2.68	1.61
	Acetyl	Acetyl	Acetyl
	Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-Lys-Arg-	5	10
Position in peptide			
Position in protein	(5)	(10)	(15)

Fig. 3.6.3 : Sites of in vitro acetylation of lysine residues in monoacetylated peptide T(M)-2. (Data obtained from Fig. 3.5.2.2.1).

In all lysine positions in which significant radioactivity was present the radioactive compound co-migrated with the PTH-derivative of ϵ -N-acetyllysine on thin layer chromatography.

The significantly acetylated lysine sites in both histones H3 and H4 occurring under in vitro conditions are the same as those reported to undergo acetylation in vivo (see Table 1.2.7). This indicates a fair degree of retention of the substrate recognition properties of the enzyme(s) and also suggests a substrate conformation near the acetylation sites closely resembling that existing in vivo. The observation that residue 9 in histone H3 shows the lowest degree of acetylation (Fig. 3.6.1) may be due to the fact that this particular lysine residue, in a wide variety of organisms, is also consistently methylated to a fairly high degree (Brandt et al., 1974a).

Sequential degradation of mono-acetylated T(M)-2 revealed that none of the lysine residues was preferentially acetylated to the exclusion of the others (Fig. 3.6.3), indicating that mono-acetylated histone H4 contains several species of mono-acetylated polypeptide. Fig. 3.6.3 shows that lysine in mono-acetylated histone H4 has been acetylated by the enzyme in either the 5, 8 or possibly 12 position indicating that the acetylation enzyme(s) probably recognises the immediate neighbourhood of a lysine residue and that acetylation of these sites occurs independently of each other, rather than in a cascade-type effect where mono-acetylation is required before di- or tri-acetylation of histone H4 can occur.

A comparison of the three major acetylation sites in histone H4 (residues 5, 8 and 12) and the major site in histone H3 (residue 14) shows that these residues all have a similar environment, namely Gly-Lys-X where X may be either glycine or alanine. Three of the four remaining acetylation sites in histone H3 (lysines 4, 9 and 23) have an environment where either serine or threonine immediately precede or follow acetyllysine. These classes of acetylation environment have previously been noted by Dixon (Dixon et al., 1975a). Lysine residue 16 in histone H4 and residues 9 and 18 in histone H3 are members of an Arg-Lys or Lys-Arg pair (DeLange et al., 1972). These differences in the acetylation sites may well indicate the existence of several acetylation enzymes in the crude histone transacetylase enzyme extract used for this study.

This study has shown that a histone transacetylase preparation can be obtained which retains a fair degree of its substrate specificity and

it would be interesting to investigate whether this preparation contains more than one histone transacetylase or, alternatively, to incubate this enzyme preparation with histone preparations which are reported not to contain specific histone aggregates after denaturation, for example by acid or guanidium chloride treatment (Kornberg and Thomas, 1974; Roark et al., 1976) and to see whether the same acetylation patterns result, which would help to answer the question of whether the histone transacetylases are sequence or conformation specific with respect to the recognition of acetylation sites in their substrates.

The role of histone acetylation in DNA replication is discussed below in the context of the current concept of chromatin consisting of subunits (Fig. 3.6.5). This was suggested by two recent models (Dixon, 1976; Hewish, 1976) which postulated the involvement of these subunits in DNA replication. Histone acetylation could however also have other functions in the cell (see 1.2.7).

Dixon has postulated that the histone aggregate is bound to DNA via the basic N-terminal regions of the histones and that acetylation occurs to loosen the DNA-histone interaction. The replication fork can then pass without disruption of the nucleosome. Deacetylation then "locks" the histone aggregates back onto the DNA (Dixon, 1976).

Ideas on the 'ground state' of chromatin before DNA replication begins have undergone radical changes fairly recently. The supercoil model for the structure of chromatin (Richards and Pardon, 1970) has recently been succeeded by models which postulate (despite some discrepancies in the reported size of the particle and the length of DNA contained in it) that chromatin is composed of subunits (Van Holde et al., 1974, 1975a, 1975b; Kornberg, 1974; Baldwin et al., 1975; Weintraub et al., 1975; Li, 1975; Pardon et al., 1975; Finch and Klug, 1976; Hewish, 1976). These subunits have been called PS particles (Rill and Van Holde, 1973), v-bodies (Olins and Olins, 1974) and more commonly nucleosomes (Oudet et al., 1975). These models propose that the nucleosome is composed of a core particle comprising histones H2A, H2B, H3 and H4 which are specifically complexed together, with the DNA wound around the outside of this core. The data currently known about histone-histone complex formation is summarised in Fig. 3.6.4.

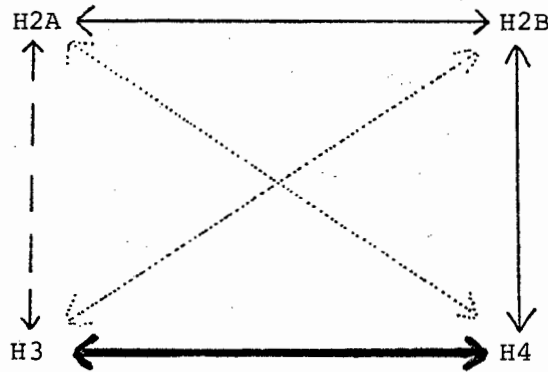


Fig. 3.6.4 : Histone cross complexing pattern. Solid lines show very strong complexes. Dashed line shows a weaker complex, while dotted lines show considerably weaker complexes (from Van Holde and Isenberg, 1975b).

It has been proposed that the hydrophobic regions of the histones interact to form a compact core with the basic N-terminal regions protruding (Van Holde et al., 1974; Baldwin et al., 1975; Roark et al., 1976). The N-terminal region of histones H3 and H4 has been postulated to be the main DNA binding site (DeLange et al., 1969a, 1972), and indeed all of the radioactivity in these histones occurred in this region (Figs. 3.6.1 and 3.6.2). As Fig. 3.6.4 shows all of the "core" histones can interact with one another to varying degrees. Histone H1 is thought to be separate from the core and to have another function in chromatin. The determination of which histone-histone complexes exist in vivo and of the stoichiometry of the histone subfractions is still under investigation in many laboratories, however Van Holde has recently stated that "the stoichiometric data are generally consistent with a repeat (in the nucleosome) in the range of 150-200 DNA base pairs, but do not in themselves indicate how the histones are distributed over the subunit" (Van Holde and Isenberg, 1975b). It has been proposed that each nucleosome contains two of each of the "core" histones H2A, H2B, H3 and H4 with either a H3-H4 tetramer or a tetramer composed of all four core histones as the main component (Van Holde et al., 1974; Van Holde and Isenberg, 1975b; Kornberg, 1974; Kornberg and Thomas, 1974; Thomas and Kornberg, 1975; Weintraub et al., 1975; Li, 1975; Pardon et al., 1975).

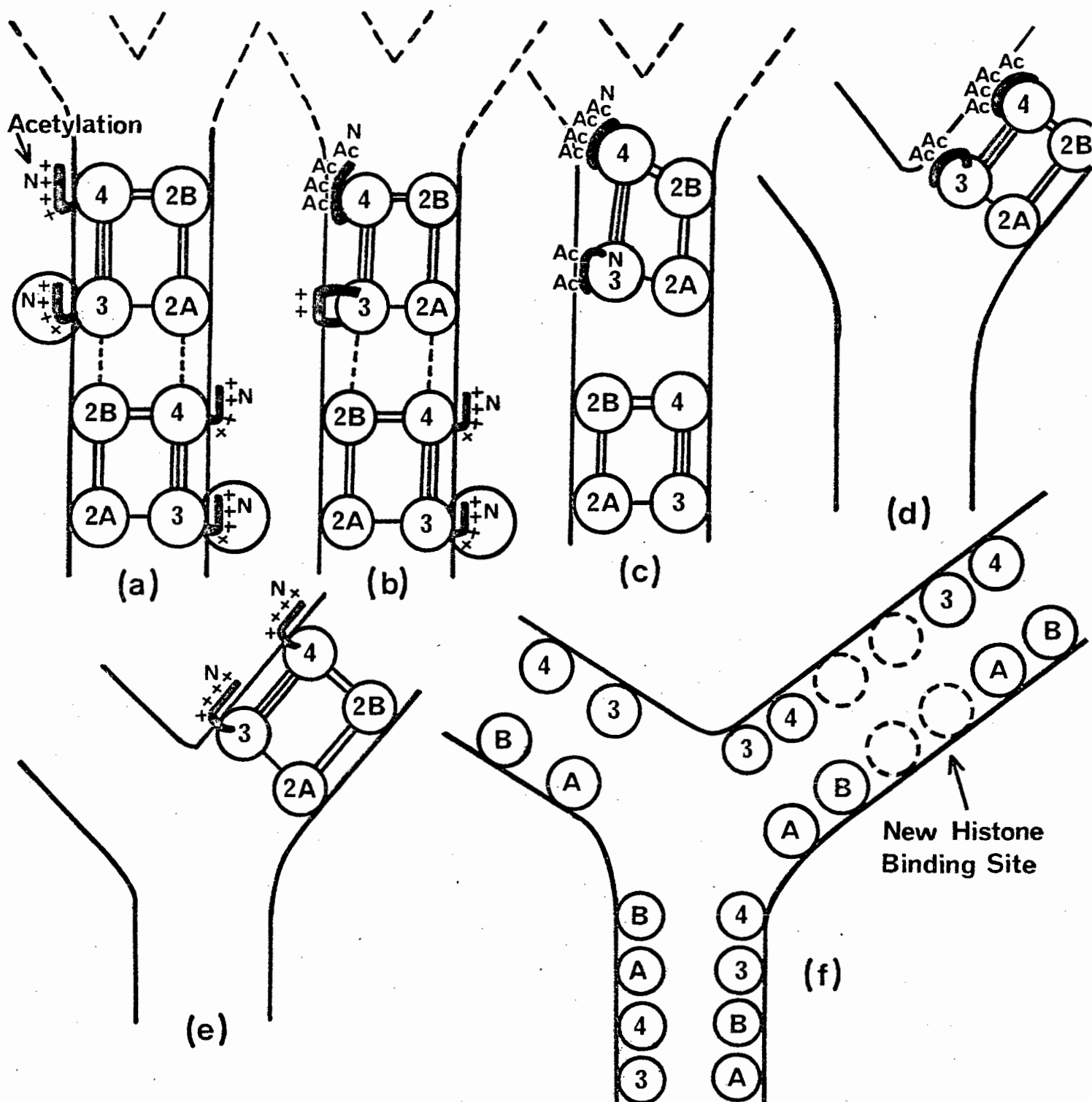


Fig. 3.6.5 : Speculative model for involvement of histone acetylation in DNA replication (Dixon, 1976; Hewish, 1976).

- (a) Chromatin in "ground state" showing one nucleosome consisting of an octamer
- (b) Histone H4 acetylated and removed from DNA with simultaneous exposure of H3 acetylation sites.
- (c) Acetylation of H3 and removal from DNA of tetramer. DNA strand separation.
- (d) Passage of replication fork and localisation of poly-acetylated N-termini on new DNA strand.
- (e) Deacetylation of 'old' histones to lock them into place on DNA.
- (f) Chromatin after 3rd acetylation cycle. 'New' histones find binding sites on daughter DNA helices.

— DNA; N-terminal histone region; 2a, 2B, 3 and 4 - histones
Ac - ε-acetyl group.

As mentioned above, the actual arrangement of the histones within the nucleosome is still under intensive investigation in many laboratories. Conflicting reports of the distribution of histones during DNA synthesis also exist. Tsanev et al. (1974) have proposed that during DNA replication the "old" histones remain bound to the parental DNA strands while the newly synthesised histones become bound to the new DNA strands. These authors also suggested that "the specific arrangement of histones on DNA is maintained during replication by means of specific interactions between pre-existing DNA-bound histones and newly synthesised free histones" (Tsanev et al., 1974). Jackson et al. (1975) concluded however that random deposition of new histones occurs onto both the parental and replicated strands of DNA but they "do not exclude the possibility that histones are deposited in a highly organised manner at the replication fork and that they then subsequently randomise". It is also possible that some histone subfractions remain on the "old" DNA while other pre-existing histone subfractions can move to the new strands since neither of these groups of workers investigated the distribution of individual histone subfractions during DNA replication. A model for DNA replication in eukaryotes which postulates the specific involvement of chromatin subunits (Hewish, 1976) also predicts that one strand of the replicating DNA helix should remain associated with pre-existing chromatin subunits during and after replication.

The model shown in Fig. 3.6.5 is a highly diagrammatic representation of an extension of Dixon's model (Dixon, 1976). A fundamental assumption made is that during DNA replication only the N-terminal regions of histones H3 and H4 are removed from the DNA, since these histones were found to be the fractions which were the most acetylated (Fig. 3.3.3.1.1). As these histones form the strongest histone-histone complex it is also unlikely that the H3-H4 complex will be disrupted during replication.

All of the major sites of acetylation in H4 (residues 5, 8 and 12) and H3 (residue 14) have a similar environment namely Gly-Lys-X (X=Ala or Gly), which points to the possibility of the same histone transacetylase causing acetylation at these sites. It is proposed that in each histone H3-H4 pair the two N-terminal regions are attached to the same DNA strand (Fig. 3.6.5 (a)) to facilitate acetylation by the histone transacetylase.

If eight histone molecules are contained in each nucleosome as reported

then two H3-H4 pairs must also be present. It would assist acetylation by the histone transacetylase if the N-terminal regions of these histones were fairly close to one another on the surface of the nucleosome, but on different DNA strands. This would aid recognition of the correct binding sites on DNA for the newly synthesised histones (see Fig. 3.6.5.(f)). In Fig. 3.6.5.(a) the H3-H4 pairs are not shown close together but this could be achieved by supercoiling of the DNA helix. Fig. 3.6.5.(a) also indicates that the entire N-terminal region of histones H3 and H4 are bound to DNA. The N-terminal group of histone H4 is acetylated and is not able to bind to the DNA. The four ϵ -amino groups in this region can all undergo acetylation as a decreasing gradient of acetylation was found from the N- to the C-terminus (Fig. 3.6.2). Multiple acetylation of this region could thus account for the removal of the N-terminal region of H4 from DNA. However histone H3 has a free α -amino end group as well as two lysine ϵ -amino groups at residues 4 and 9 which are only minor sites of acetylation (Fig. 3.6.1). It is proposed that the decreasing gradient of acetylation of H3 from residue 14 onwards towards the C-terminus (Fig. 3.6.1) could not alone account for the removal of the H3 N-terminal region from the DNA. If histone H4 acetylation occurs prior to acetylation of H3 (Fig. 3.6.5.(b)), then after the N-terminal region of histone H4 is removed from DNA, a conformational change in the H4-H3 complex could occur (Fig. 3.6.5.(b)). This conformational change could remove the N-terminal amino group as well as lysine 4 and/or 9 from DNA, for example by bringing carboxyl groups close to these residues, which would then allow acetylation of histone H3 to occur with the resulting removal of the H3 from the DNA.

If the histone H2A and H2B molecules are non-acetylated they could remain bound to the pre-existing DNA and allow the histone complex to swing out away from one of the DNA strands and allow DNA strand separation and replication to occur (Fig. 3.6.5.(c and d)).

The acetylated histone groups can then find their binding sites on the new DNA (Fig. 3.6.5.(d)) and be deacetylated and locked into place as suggested previously (Louie et al., 1973; Dixon, 1976) (Fig. 3.6.5(e)).

Newly synthesised histones could then be guided to their specific binding sites as proposed by Tsanev et al. (1974). This could be directed via specific hydrophobic interactions with the old histones, as well as with

the histone binding sites which exist on the DNA between the DNA-bound histone complexes (Fig. 3.6.5(f)).

PART 4

MATERIALS AND METHODS

4.1 MATERIALS

Double glass-distilled water was used to make up all solutions. Deionised water was used for dialysis.

Calf thymus was obtained from the local abattoir approximately 30 min after slaughter, packed in ice and transported to the laboratory.

Coenzyme-A was obtained from Miles Laboratories (Cape Town) or P-L Biochemicals (Wisconsin, U.S.A.).

³H-acetic anhydride was purchased from the Radiochemical Centre, Amersham, England.

o-Iodosobenzoic acid was supplied by Pierce Chemical Co.

CM-cellulose (CM52 microgranular, preswollen), was obtained from Whatman. Different grades of Sephadex and Sepharose were obtained from Pharmacia (Uppsala, Sweden).

DEAE-cellulose (medium mesh, 0.8 meq/gm) was obtained from Sigma Chemical Co. Biogel was supplied by Biorad (Richmond, California).

α-chymotrypsin (48 units/mg) and DCC-treated trypsin (bovine type XI, 9000 units/mg) were supplied by Worthington Biochemicals and Sigma Chemical Co. respectively.

Thrombin (purum grade, 300 NIH units/mg protein) was supplied by Behringwerke (Marburg-Lahn).

Preparation of PTH- ϵ -N-acetyllysine

ϵ -N-acetyllysine (0.25 mmoles, Aldrich Chemical Co.), was coupled with PITC as described by Edman (1970), except that as only 0.25 mmoles of amino acid was coupled, the concentrations of acid and alkali solutions were decreased from 2 N to 0.02 N. Conversion of the PTC- ϵ -N-acetyllysine and crystallisation of the product were performed exactly as described by Edman (1970). Consumption of alkali during coupling indicated that 0.25 mmoles of amino groups had been coupled.

A $\frac{\sum_{245 \text{ nm}}}{\sum_{269 \text{ nm}}}$ ratio of 0.378 to 0.429 indicated that no PTH-PTC lysine

was present (Edman, 1970).

Preparation of ^3H -acetyl CoA

The preparation of ^3H -acetyl CoA using the method of Stadtman (1957) was done twice using ^3H -acetic anhydride with specific activity of 2.06 or 5.4 Ci/mmole. The preparation with the higher specific activity was used for the enzymatic preparation of ^3H -acetylhistone described in Part 3, while the ^3H -acetyl CoA with the lower specific activity was used for the assays done during the purification and characterisation of the enzyme.

To prevent losses due to the volatility of ^3H -acetic anhydride the vial was warmed to room temperature and the tip then dipped into liquid nitrogen for at least 30 min prior to opening. Coenzyme A (CoASH) was dissolved at a concentration of 10 $\mu\text{mole/ml}$ in distilled water at 0°C and the pH adjusted to 7.5 with 1 M KHCO_3 . This solution was added to the vial containing ^3H -acetic anhydride immediately after opening. The reaction mixture was allowed to stand for 5 min at 0°C , after which time the pH was then adjusted to 6.0 with HCl and the reaction mixture applied to a Sephadex G-10 column. Fig. 4.1.1 shows the typical elution profile obtained.

Fraction I is ^3H -acetyl CoA since it has the greater molecular weight of the two peaks and is a cofactor for histone transacetylase activity, i.e. when incubated with sucrose gradient histone transacetylase fractions (4.4.2) and histone substrate, incorporation of ^3H -acetyl groups into

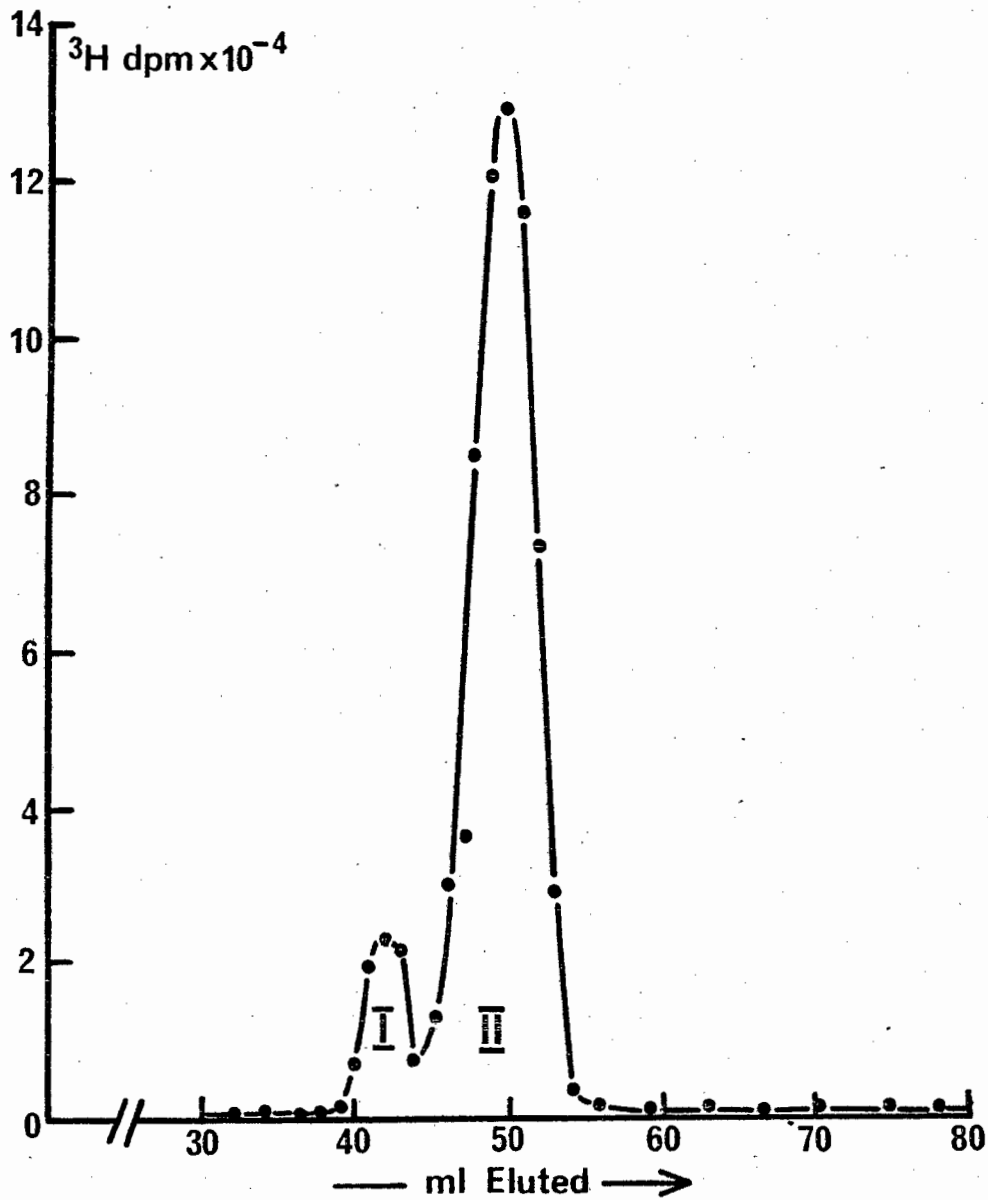


Fig. 4.1.1 : Separation of ^3H -AcCoA (peak I) from ^3H -acetic acid (peak II). Reaction mixture of ^3H acetic anhydride and CoASH applied to a Sephadex G-10 column (1.5 x 66 cm, gel bed volume 117 ml) equilibrated and eluted with H_2O (pH 6.0). Fractions of 1 ml were collected. ^3H -dpm values of 5 μl aliquots taken for counting are plotted above.

histone occurs. Fraction II is ^3H -acetic acid since it has a smaller molecular weight and is not a cofactor for histone transacetylase activity. Table 4.1.1 summarises the preparation of ^3H -acetyl CoA.

TABLE 4.1.1

SUMMARY OF PREPARATION OF ^3H ACETYL CoA

Preparation number	1	2
Specific activity of ^3H -acetic anhydride	2.06 Ci/mmole	5.4 Ci/mmole
Amount acetic anhydride (micromoles)	12.14 (25 mCi)	4.63 (25 mCi)
Amount CoASH in reaction mixture (micromoles)	10.7	3.3
^3H -Ac CoA recovery	2.1 mCi	2.06 mCi
% of theoretical yield	17%	17%
Total ^3H -recovery as (AcCoA + HAC) from Sephadex G-10	56% (14 mCi)	60% (15 mCi)

The low yield could be due to loss of ^3H -acetic anhydride either by volatilisation or reaction with the water in the reaction mixture.

4.2 GENERAL METHODS

Freeze-dried protein samples were stored at -22°C in glass vials with screw-on plastic lids.

Column chromatography was followed by OD measurements at 206 nm and 280 nm or 260 nm with a Uvicord III (L.K.B., Sweden).

4.2.1 Protein determination

Protein was determined spectrophotometrically by the method of Lowry et al.

(1951) using crystalline bovine serum albumin (Miles) as a standard. Interfering substances such as mercaptoethanol, Tris buffer or sucrose were removed by overnight dialysis against distilled water.

4.2.2 Amino end group determination with DANSYL chloride

The method described by Gray (1972) was used to determine N-terminal amino groups. Protein (50-250 µg) was dissolved in 50 µl 1% sodium dodecyl sulphate by heating in a boiling water bath for 2-5 min. After cooling 50 µl of N-ethylmorpholine and 100 µl of dansyl chloride (25 mg/ml in dimethylformamide, freshly prepared) were added and incubated for 1 h at room temperature. Acetone (0.5 ml) was added to precipitate the protein and the pellet collected by low speed centrifugation was washed with 0.5 ml of 80% acetone. The pellet was then dried, 0.1 ml of 5.7 N HCl added and the tubes were sealed and incubated at 110°C for 4 h. The tubes were cooled before opening, dried in a vacuum desiccator and the contents extracted with 0.2 ml H₂O-saturated ethyl acetate. The dried residue was dissolved in 5 µl acetone-acetic acid (3:2) and spotted onto a 5 x 5 cm micropolyamide TLC-Ready plastic sheet (Schleicher and Schüll Fl700). The plates were developed in 2 dimensions, first with H₂O:formic acid = 200:3 and then with benzene:acetic acid = 9:1. The spots were then visualised in an Ultraviolet light box.

4.2.3 Reinecke salt precipitation of histones

Histones dissolved in 0.25 M HCl were occasionally precipitated with Reinecke salt ($\text{NH}_4(\text{Cr}(\text{NH}_3)_2(\text{SCN})_4) \cdot \text{H}_2\text{O}$) according to the method of Lindh and Brandtmark (1960).

An equal volume of saturated Reinecke salt solution (1.6 g/100 ml at 4°C) was added to the histone solution and allowed to stand for 30 min at 4°C. The resulting precipitate after low speed centrifugation was washed twice with 1 ml of 0.05 M Tris-HCl (pH 8.5) to remove contaminating non-histone proteins and resuspended in 0.25 M HCl. An equal volume of saturated Reinecke salt solution was added and allowed to stand for 5 min at 4°C. The precipitate after centrifugation was washed once with ice-cold water and then extracted twice with acetone : 2 N HCl (98:2) which cleaved the histone Reineckate, extracts the Reinecke acid and leaves the free histone hydrochloride as the precipitate.

4.2.4 Radioactivity determination

Radioactivity was determined in a Beckman model LS-250 liquid scintillation counter. Counting efficiency for ^3H was approximately 37%. Counts per minute (cpm) were converted into disintegrations per minute (dpm) by means of suitable quench correction curves stored in a bench-top computer (Wang 700) programme. Counting was done in plastic-topped glass scintillation vials using 10 ml of scintillation solution. If samples were oxidised in a Packard Tri-Carb sample oxidiser the radioactivity was trapped in 10 ml of Monophase 40 (Packard) scintillator. For all other samples 10 ml of toluene-based scintillator solution containing 10% BBS-3 solubiliser (Beckman Inst.), 0.5% PPO (2,5-diphenyloxazole), 0.01% POPOP (2,2'-p-phenylene bis(5-phenyloxazole) was used.

4.2.5 Gel electrophoresis of histones

Polyacrylamide gel electrophoresis of histones was performed in 15% acrylamide gels containing 2.5 M urea, according to the method of Panyim and Chalkley (1969) using either 10 or 20 cm long gels.

The following solutions were prepared :

- A. 60% (w/v) acrylamide, 0.4% (w/v) N,N'-methylenebisacrylamide
- B. 4% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) in 43.2% (v/v) glacial acetic acid
- C. 0.2% (w/v) ammonium persulphate, 24% (w/v) urea.

All solutions were stored at $+4^{\circ}\text{C}$, except C which was prepared just before use. Pre-treatment of the glass tubes with 0.5% dimethyldichlorosilane in toluene, improved the menisci of the 20 cm gels and facilitated their removal from the tubes.

Gels were prepared by mixing A, B and C in the volume ratio 2:1:5. This solution was degassed under vacuum in a side-arm flask for 1-2 min with shaking, and quickly poured into 10 x 0.5 cm or 20 x 0.5 cm glass tubes, which were sealed at the bottom with Parafilm (American Can. Co. or Gallenkamp). After water had been carefully layered over the gel solution which had been poured to within 1 cm of the top of the tubes, polymerisation was allowed to occur for 1-2 h at room temperature.

The 10 cm gels were pre-electrophoresed in a disc gel electrophoresis apparatus (Shandon) using 0.9 M acetic acid (pH 2.5) as tray buffer, at 2 mA per tube for 5 hours until the voltage remained constant (about 160 V). The 20 cm gels were pre-electrophoresed at 1.2 mA/gel for 9 hours with a final voltage of 280 V being reached.

The sample was dissolved at a protein concentration of 1 mg/ml, in freshly prepared 8 M urea - 0.5 M mercaptoethanol and allowed to stand at room temperature for at least 1 hour before application.

At the end of pre-electrophoresis the tray buffer was changed, sample (10-50 µg) applied and electrophoresis performed for 3½ hours for the 10 cm gels or for 12 hours for the 20 cm gels.

Gels were removed from the tubes by cooling in ice and forcing water between the gel and the glass tube with the aid of a syringe. Gels were stained with 0.1% amido black (Merck) - 7% (v/v) acetic acid for ½ h and destained with 7% acetic acid in a transverse disc destainer for 1 h, or else by diffusion over 2 days with changes of destaining solution every 8 hours. Gels were stored in 20% ethanol - 7% acetic acid at +4°C in corked tubes.

Densitometric tracings of gels were obtained with a Vitatron TLD 100 densitometer using 615 nm filter.

4.2.6 Determination of ^3H in polyacrylamide gels

Polyacrylamide gels after staining and destaining were cut into 1 mm slices using a gel slicer. The slices were then subjected to either :

- a) Air drying at 40°C in an oven for 1 hour, followed by oxidation in the sample oxidiser and counting; or in earlier experiments
- b) Solubilisation in a counting vial at 60°C for 3 hours with 0.1 ml of 30% H_2O_2 (Grower and Bransome, 1970) followed by the addition of 0.4 ml of a 50% saturated solution of ascorbic acid. Scintillation solution was then added and the samples stored in the dark for 12 h to decrease any chemiluminescence still present.

4.3

ASSAY OF HISTONE TRANSACETYLASE ACTIVITY

Assays of histone transacetylase activity in routinely isolated fractions were performed as described below unless otherwise stated. In every case corresponding buffer blanks were incubated to correct for non-enzymatic acetylation (see 2.3). Incubations to determine enzymatic acetylation were done in triplicate while non-enzymatic incubations were done in duplicate. 1 enzyme unit was defined to be equivalent to 1 pmole of acetate incorporated into histone under the incubation conditions used.

Solutions used for incubations

- a) 0.25 M sucrose - 0.05 M Tris-HCl (pH 7.9) - 0.025 M KCl - 1.5 mM MgCl_2 (0.25 M sucrose - TKM)
- b) 0.14 M Tris-HCl (pH 7.9) - 0.25 M KCl - 3.5 mM MgCl_2 (MKT)
- c) Histone solution (12.5 mg calf thymus total histone (acid extracted) dissolved in 1 ml of water, i.e. 0.08 ml contains 1 mg histone).
- d) ^3H -acetyl CoA solution containing 0.02 $\mu\text{Ci}/10 \mu\text{l}$ (specific activity 1.03 Ci/mmol AcCoA)
- e) 0.3 M NH_4Cl - 0.03 M Tris-HCl (pH 7.9) - 1 mM EDTA - 5 mM 2-mercaptoethanol (0.3 M NH_4Cl - TEM)

Assay for nuclei :

Nuclei suspended in 0.4 ml of 0.25 M sucrose-TKM were incubated with 0.1 ml MKT, 0.08 ml histone solution and 10 μl of ^3H AcCoA solution.

Assay for crude histone transacetylase extract :

13 μl crude extract in 0.3 M NH_4Cl -TEM, 78 μl 0.3 M NH_4Cl -TEM, 0.309 ml H_2O , 0.1 ml MKT, 0.08 ml histone solution and 10 μl ^3H AcCoA solution were incubated.

Assay for sucrose gradient fractions :

As for crude extract except the 13 μl of enzyme preparation is in addition approximately 14% w.r.t. sucrose.

When sucrose gradients were run at different concentrations of NH_4Cl (Fig. 2.2.2.1) fractions were dialysed against 0.01 M NH_4Cl -TEM and histone transacetylase activity was assayed by incubating together 0.13 ml of gradient fraction (in 0.01 M NH_4Cl -TEM), 0.27 ml 0.01 M NH_4Cl -TEM, 0.1 ml MKT, 0.08 ml H_2O containing 1 mg calf thymus histone (acid extracted) (4.5.1), and 10 μl containing 0.02 μCi ^3H AcCoA (specific activity 1.03 Ci/mmmole).

Sucrose gradient fractions were used to determine the substrate specificity of various histone preparations (Tables 2.6.2 and 2.6.3).

The incubation constituents used were (a) Enzyme : Undialysed : 90 μl (45 μg protein) sucrose gradient fractions; dialysed (vs. 0.01 M NH_4Cl -TEM) : 120 μl (45 μg protein) + 10 μl of 2.63 M ammonium sulphate to bring ionic strength of incubation to 0.122. Buffer blanks : 90 μl of 0.3 M NH_4Cl -TEM or 120 μl of 0.01 M NH_4Cl -TEM + 10 μl of 2.63 M ammonium sulphate. (b) Substrates : I. Calf histone : 500 μg in 0.08 ml water; II. Rat histone (acid extracted) : 500 μg in 0.16 ml water; III. Histone subfractions : Amounts indicated dissolved in 0.07 ml water; IV. Subfractions combined : 0.07 ml of each fraction; V. Protamine displaced histone : 500 μg in 0.16 ml of water; VI. Chromatin : containing 500 μg histone in 0.34 ml water. (c) Other constituents : 0.1 ml MKT buffer plus water to the final incubation volume of 0.58 ml and 10 μl containing 0.02 μCi of ^3H AcCoA.

When chromatin was used as substrate the dialysed enzyme preparation was added to the chromatin gel and pre-incubated for 10 min at $+4^\circ\text{C}$, after which the ^3H AcCoA was added and the gel well mixed on a vortex mixer. The rest of the incubation constituents were then added (which caused immediate precipitation) and the tubes incubated at $+37^\circ\text{C}$. The reaction was terminated after 30 min by acidification as described below.

For all the other substrates the reaction was initiated by the addition of labelled acetyl CoA and terminated by acidification as described below. In the case of the histone subfraction incubations (Table 2.6.3.III), 400 μg of calf thymus total histone (acid extracted) was added as carrier after acidification but before acetone precipitation. ^3H -acetyl histone samples were washed and the radioactivity determined as described in 4.3.

Assay for DEAE-cellulose fractions :

Since DEAE-cellulose was eluted with a 0.01 - 0.3 NH_4Cl gradient, decreasing volumes (y ml) were assayed with increasing salt concentration in order that the ionic strength of the incubation be kept constant and equal to that of the fractions above, i.e. ionic strength of 0.122. The $[\text{NH}_4\text{Cl}]$ of the column fractions was determined by Nesslerisation as described by Vogel (1961). y ml of DEAE-cellulose fractions in 0.01 to 0.3 M NH_4Cl -TEM, (0.4-y) ml H_2O , 0.1 ml MKT, 0.08 ml histone solution and 10 μl ^3H AcCoA solution were incubated.

Assay for Sephadex G-200 or Sepharose 4B column fractions :

Incubations were the same as for the crude extract above.

Initiation and termination of reaction and preparation of histones for counting :

Incubation tubes containing all constituents were kept at $+4^\circ\text{C}$ until the labelled compound was added, when the tubes were mixed on a vortex mixer and placed in a water bath at $+37^\circ\text{C}$ for 30 min. The reaction was terminated by the addition of 2 M HCl to a final concentration of 0.25 M, mixing and immediate cooling to 4°C . In the case of nuclei the histones were extracted with 0.25 M HCl for 30 min at $+4^\circ\text{C}$ at the end of the incubation period after which time the samples were then subjected to low speed centrifugation and the supernatant containing the histones subjected to further processing. To the clear acidic solutions 10 volumes of ice-cold acetone were added and the samples were stored at -22°C for approximately 12 hours to precipitate the histones as described by Pogo et al. (1966). The precipitates were then recovered by centrifugation, resuspended as described by Noland et al. (1971) in 2 ml of 18% (w/v) trichloroacetic acid (TCA) and heated for 15 min at 90°C to hydrolyse any nucleic acids or polysaccharides which may have co-precipitated from the acetone with the histones. The precipitate was then washed twice with 1 ml of 18% TCA and once with 1 ml methanol-ethanol-ether = 2:2:1 (ratio by volume) to remove any lipid material and TCA. The purified histones were then redissolved in 0.5 ml of 0.25 N HCl and their radioactivity determined.

4.4 PREPARATION OF HISTONE TRANSACETYLASE

4.4.1 Isolation of nuclei

Liver nuclei were isolated from male Wistar rats (150-200 g). Rats were starved overnight and killed and the livers excised, trimmed and weighed (about 120 g liver).

A 40% (w/v) homogenate in ice-cold 0.25 M sucrose-TKM was prepared in a motor-driven Potter-Elvehjem glass homogeniser by 3 or 4 passes with a teflon pestle. Connective tissue was removed by filtering through 4 layers of butter muslin and the filtrate centrifuged at 600 g for 20 min (SS-34 Sorvall rotor, 2 250 rpm). The supernatant was decanted, the pellet resuspended in 2.3 M sucrose-TKM and the total volume of 320 ml suspension was centrifuged at 45 000 g for 70 min (SS-34 rotor, 19 000 rpm). The pellet of nuclei was washed by centrifugation at 600 g for 10 min with 85 ml and then 40 ml of 0.25 M sucrose-TKM.

4.4.2 Preparation of crude histone transacetylase extract from nuclei

Washed nuclei (4.4.1) were suspended in 30 ml 0.25 M sucrose (no TKM) (Albrecht, 1973) and sonicated (Biosonik III ultrasonicator, Bronwell Scientific, U.S.A.) for 60 seconds in 30 second bursts at 2.5 amps (Large tip at 80% setting, 300 watts delivered). Since it has been found (Davies and Walker, 1974) that chromatin has a minimum solubility at 0.15 M NaCl, the solution was then slowly adjusted with stirring to a higher ionic strength by the addition of 3 ml of 1.4 M NaCl - 0.01 M MgCl_2 - 0.5 M Tris-HCl (pH 8.0). The dense white deoxynucleoprotein precipitate which immediately formed was removed by centrifugation at 7 200 g for 30 min. The supernatant was then freeze-dried and the dried material redissolved in 5 ml 0.3 M NH_4Cl -TEM and dialysed against the same buffer. The density of the extract was checked to ensure that it was less than that of 5% sucrose - 0.3 M NH_4Cl -TEM. Any particulate matter or insoluble nucleohistone was removed by centrifugation at 198 000 g (50K rpm, 50 rotor, Beckman) for 15 min. The supernatant (crude extract) was stored in 600 μl aliquots in sealed Eppendorf micro-centrifuge plastic tubes in liquid N_2 if not used immediately.

4.4.3 Sucrose gradient ultracentrifugation

4.4.3.1 Sucrose gradient formation

I. SW 65 Rotor :

Polyallomer or cellulose nitrate tubes (1.3 x 5 cm) were filled with sucrose gradients using a Beckman density gradient former (Beckman Instruments) to within 0.5 cm of the top of the tubes.

II. SW 40Ti Rotor :

Since the Beckman density gradient former can only fill 3 tubes and the SW 40 rotor has 6 buckets, sucrose gradients for the SW 40 were formed as described below. An 8-channel peristaltic pump (Scientific Manufacturing Co., Cape Town), conical flask and magnetic stirrer were set up as shown in Fig. 4.4.3.1. The inlet lines that carry the denser sucrose solution to the mixing chamber had a combined delivery rate equal to half of the combined removal rate of the 6 identical outlet lines to the ultracentrifuge tubes. 6 cellulose nitrate SW 40 rotor tubes (1.4 x 9.5 cm) were graduated to 12 ml and placed vertically in crushed ice. The inlet and outlet lines were primed with dense- and light sucrose solutions respectively and allowed to run to drain. The pump was then stopped and the outlet lines removed from the light sucrose solution. After the pump had been re-started and a bubble of about 5 cm allowed into each outlet line the pump was again stopped. 48 ml of light solution as well as the inlet and outlet lines were then placed in the mixing chamber. The magnetic stirrer and pump (delivery rate 0.8 ml/outlet tube/min) were then started with the outlet lines running to drain until the air bubbles marking the start of the gradient had been cleared from the lines. The pump was then stopped and the glass capillary tubes placed in the SW 40 tubes and the gradient then allowed to form by restarting the pump. As each tube filled to the 12 ml mark, the pump was stopped while the outlet line was removed from that tube. This is necessary as the peristaltic tubing used has a small variation in flow rate from tube to tube.

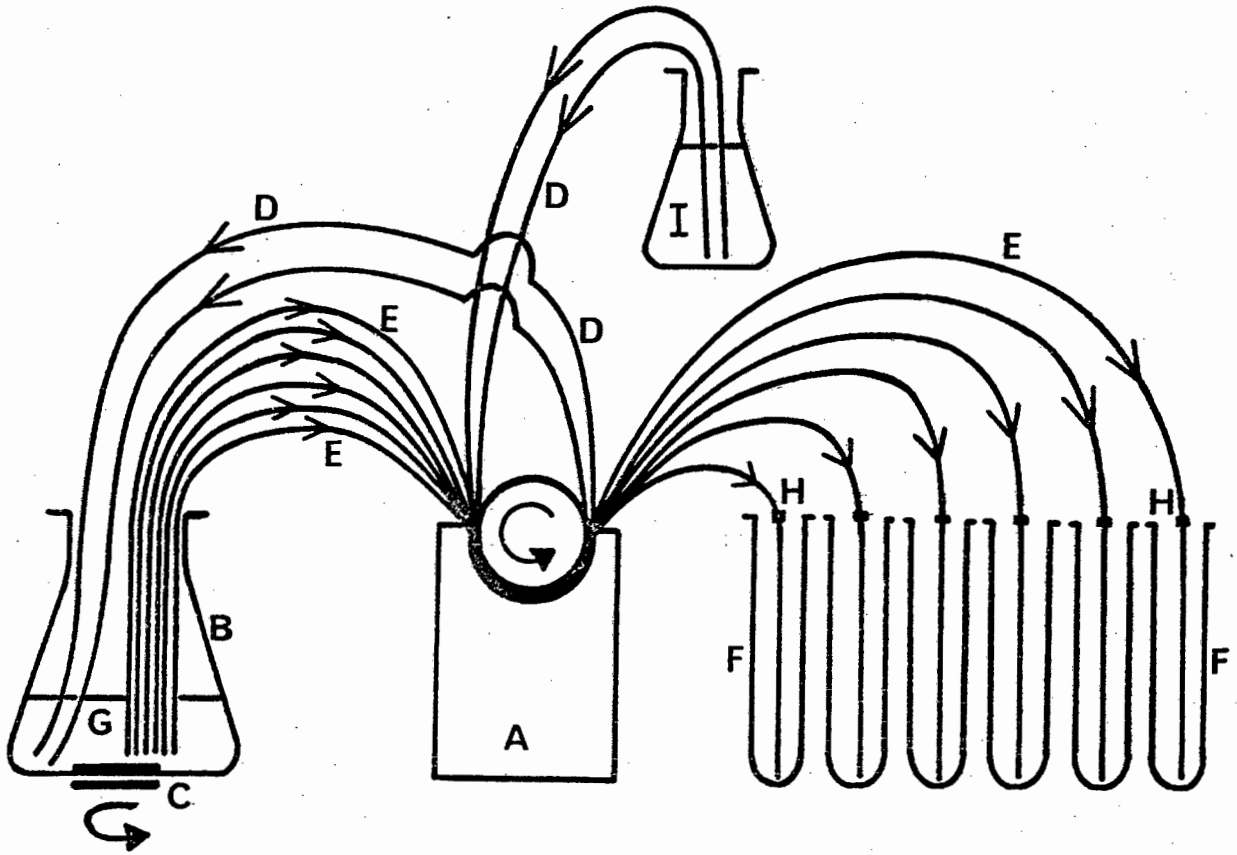


Fig. 4.4.3.1 : Gradient forming apparatus.

- A. 8-channel peristaltic pump
- B. Conical flask mixing chamber
- C. Magnetic stirrer
- D. Inlet lines (2) from dense sucrose solution
- E. Outlet lines (6) to tubes
- F. 6 x SW40 rotor tubes
- G. Sucrose solution of low density
- H. Thin walled glass capillary tubes
- I. Sucrose solution of high density

Directions of flow as indicated. All containers were kept on ice.

Solutions used for gradient formation :

Dense sucrose solution : 25% sucrose - 0.3 M NH_4Cl -TEM

Light sucrose solution : 5% sucrose - 0.3 M NH_4Cl -TEM

4.4.3.2 Application of sample and ultracentrifugation

600 μl of crude extract (4.4.2) were carefully layered on top of each gradient with an all-glass 1 ml syringe. Centrifugation was performed at $+4^\circ\text{C}$ at 420 000 g (65K rpm), SW 65Ti rotor, or 284 000 g (40K rpm), SW 40Ti rotor in a Beckman model L2-75B ultracentrifuge for times indicated in Part 2.

4.4.3.3 Recovery of sucrose gradients

The peristaltic pump used to form the gradients was set at a speed of 1 ml/min and used to pump out the gradients by means of a capillary tube to the bottom of the tube. The OD_{230} was monitored by passing the gradient through a 0.1 cm pathlength flow-through cuvette. Fractions of 0.5 ml were collected and the fractions with histone transacetylase activity pooled.

4.4.4 Application of sucrose gradient fractions to DEAE-cellulose

Sucrose gradient fractions were dialysed against 0.01 M NH_4Cl -TEM until a Nessler determination (Vogel, 1961) of an aliquot indicated that the NH_3 concentration was 0.01 N.

DEAE-cellulose swollen and prepared for use as recommended by the manufacturers, was packed into a 2.5 x 12 cm column. The column was washed with 2 volumes of 0.3 M NH_4Cl -TEM and then with at least 2 column volumes of 0.01 M NH_4Cl -TEM before the sample was applied. A peristaltic pump was used to maintain a flow rate of 36 ml/h. The salt gradient was applied as described (see Part 2). Fractions of 3 ml were collected and those with histone transacetylase activity were pooled.

4.4.5 Storage of enzyme preparations

Enzyme preparations were stored frozen in sealed tubes in a LD-40 liquid

nitrogen container (Union Carbide, U.S.A.) above the liquid nitrogen (maximum temperature, -100°). DEAE-cellulose fractions were adjusted to 20% glycerol before storage.

4.5 ISOLATION OF HISTONES

4.5.1 Preparation of calf thymus total histone (acid extracted)

Deoxynucleoprotein (DNP) was prepared from calf thymus by repeated washes with 0.05 M NaHSO_3 - 0.09 M NaCl - 0.01 M sodium citrate (pH 6.0) as described by Busch (1968). The DNP was then extracted with 0.25 M HCl for 1 hour with stirring at $+4^{\circ}\text{C}$, centrifuged at 1 000 g and the sediment re-extracted with 0.25 M HCl. The supernatants were combined, dialysed and freeze-dried.

4.5.2 Preparation of rat liver chromatin

4.5.2.1 Insoluble chromatin suspension

Rat liver nuclei prepared as described in 4.4.1 were used to prepare chromatin by the method of van der Westhuyzen (1973) which involves lysis of the nuclei in 0.05 M Tris-HCl (pH 7.9) and centrifugation at 10 000 g for 5 min. The amount of DNA in the chromatin was determined from the absorbance at 260 nm of an aliquot of chromatin dissolved in 2 M NaCl using the formula

$$\text{OD}_{260} - \text{OD}_{320} \quad (1 \text{ mg DNA/ml}) = 22 \quad (\text{Bonner et al., 1968}).$$

An aliquot was also freeze-dried and gave a yield of 3.8 mg dry chromatin/g liver. The histone content of the chromatin was determined by acid extraction of the dried chromatin, followed by a protein determination (4.2.1) of the supernatant. This chromatin preparation was found to contain 31% DNA and 29% histone.

4.5.2.2 Chromatin gel in distilled water

The chromatin pellet in 4.5.2.1 was allowed to stand for 12-15 hours in double-distilled water at 4°C . The chromatin "swelled" to form a clear gel (van der Westhuyzen, 1973).

4.5.3 Preparation of rat liver total histone (acid extracted)

Chromatin prepared above (4.5.2.1) was extracted twice with 0.4 N H_2SO_4 for 1 h at 4°C, centrifuged, and the supernatants dialysed and freeze-dried.

4.5.4 Preparation of rat liver total histone (protamine displaced)

Protamine displaced rat liver histone was prepared from nuclei (4.4.1) by the method of van der Westhuyzen and von Holt (1971). The only modification introduced being that deoxynucleoprotein was prepared from rat nuclei by the method described in 4.5.2.1 rather than by direct extraction of calf thymus whole cells with dilute saline solutions.

4.5.5 Preparation of ^3H -acetyl rat liver total histone (protamine displaced) for sequencing studies

Optimal conditions for the incubation of sucrose gradient fractions with rat liver protamine-displaced whole histone determined in Part 2 were used.

Incubations contained : 90 μl (45 μg protein) of sucrose gradient fractions (4.4.3.3)
 0.1 ml MKT buffer
 500 μg rat liver protamine displaced histone
 in 0.33 ml H_2O
 0.04 ml H_2O
 5 μCi ^3H acetyl CoA (specific activity 2.7 Ci/mmol)

Since scaling up the entire incubation often does not give the expected proportional results, 340 incubations were done. After acidification the incubations were pooled and 10 volumes acetone was added to precipitate the histones overnight. The pellet was washed with 2 volumes of ice-cold acetone before freeze drying.

4.5.6 Histone fractionation

4.5.6.1 Calf thymus histone fractionation

Total histone isolated in (4.5.1) was used to prepare electrophoretically pure subfractions by the 2-step gel exclusion chromatography method of Böhm et al. (1973). This method utilises Biogel P-60 equilibrated and eluted with 0.02 N HCl (pH 1.7) - 0.05 N NaCl - 0.02% NaN₃ followed by Sephadex G-100 chromatography using 0.05 M sodium acetate - 0.05 M sodium bisulphite (pH 5.1) as eluant. When 2 g of total histone was subjected to this procedure, as we have reported earlier (Böhm et al., 1973), the following yields of the various histone subfractions were obtained: Histone H1 = 0.35 g, histone H2B = 0.40 g, histone H2A = 0.20 g, histone H3 = 0.22 g and histone H4 = 0.45 g. All further experimental details as well as the polyacrylamide gel electrophoretic patterns of the fractions obtained have been reported earlier by us (Böhm et al., 1973).

4.5.6.2 Rat liver histone fractionation

4.5.6.2.1 Preparation of rat liver histone H4

³H-acetyl total histone (125 mg) prepared as described in (3.2) was dissolved in 10 ml of 8 M urea - 1% mercaptoethanol and subjected to chromatography on a 5 x 88 cm column of Biogel P-60 using the method of Böhm et al. (1973). When a 50 cm H₂O pressure head was used, a flow rate of 72 ml/h was recorded. 10 µl aliquots were taken for ³H determination from the 5 ml fractions collected. Pooled fractions were dialysed and freeze dried.

4.5.6.2.2 Preparation of rat liver histone H3

Dimerisation of rat histone H3/2A/2B mixture, prepared by 4.5.6.2.1, was performed according to the method of Brandt and von Holt (1971). In a typical experiment 53 mg H3/2A/2B (approximately 1.1 µmole H3) was dissolved in 1 ml of 6 M urea which had been made cyanate free and then adjusted to pH 7.4 with 1 M Tris. The protein was allowed to disaggregate for 60 min and then 0.6 mg (2.3 µmole) o-iodosobenzoate dissolved in an equivalent amount of 0.1 N NaOH was added. The solution was then stirred

at room temperature for 3 hours, another 2.3 μ mole o-iodosobenzoate added and after a further 4 hours the reaction mixture was dialysed for 18 hours against three changes of distilled H_2O to remove any unreacted o-iodosobenzoate. An equal volume of 8 M cyanate-free urea (pH 2.5) was added and the solution allowed to stand for 5 hours. This solution was then subjected to Sephadex G-100 chromatography as described by Brandt and von Holt (1971), except that 0.02 N instead of 0.01 N HCl was used as eluant as this gave better recoveries of protein from the column.

4.6 PREPARATION OF HISTONE DEACETYLASE

3H -acetyl calf thymus total histone was prepared (4.6.1) and used to assay the histone deacetylase prepared as described below (4.6.2) during its isolation.

4.6.1 Preparation of histone deacetylase substrate

Calf thymus nuclei were prepared by homogenisation and washing of fresh tissue (100 g) with 0.25 M sucrose - 3 mM $CaCl_2$ according to the method described by Wang (1967).

Isolated nuclei suspended in 40 ml 0.25 M sucrose - 0.1 phosphate buffer were incubated with 32 ml 0.1 M glucose containing 3.75 mg NaCl/ml and 42 mg $MgCl_2$ /ml and 8 ml of water containing 0.34 mCi of 3H -acetic acid (specific activity 383 mCi/mmol) for 30 min at $37^\circ C$ as described by Gershey et al. (1968). After incubation the nuclei were washed three times, twice with 0.25 M sucrose - 3 mM $CaCl_2$ (1 mM spermidine used by Gershey et al. (1968) was substituted by 3 mM $CaCl_2$), and once in 0.14 M NaCl - 10 mM Na_3 citrate. The nucleoprotein pellet was then extracted with 0.25 M HCl and the supernatant after centrifugation at 1 000 g for 10 min was dialysed and freeze-dried. 3H -calf thymus total histone with specific activity 3 350 dpm/mg protein was obtained.

4.6.2 Isolation of histone deacetylase

Histone deacetylase was isolated by the method of Vidali et al. (1972) with the final isoelectric focussing step being omitted.

4.6.2.1 Extraction of histone deacetylase

Calf thymus (50 g) was homogenised in 50 ml 0.14 M NaCl - 25 mM Tris-HCl (pH 7.3) and centrifuged at 100 000 g for 1 h. Acetone, 3 volumes, was added to the supernatant and the precipitate was collected by centrifugation at 30 000 g for 10 min. The precipitate was extracted with 25 ml of 25 mM Tris-HCl (pH 7.3) and centrifuged at 30 000 g for 10 min. The precipitate was extracted with 25 ml of 25 mM Tris-HCl (pH 7.3) and centrifuged at 30 000 g for 10 min.

4.6.2.2 Exclusion chromatography

The supernatant was applied to a 2.5 x 92 cm Sepharose 6B column and equilibrated and eluted with 25 mM Tris-HCl (pH 7.3) at a flow rate of 45 ml/h. 10 ml fractions were collected. The eluate was monitored at 280 nm and histone deacetylase activity determined as described below (4.6.2.3).

4.6.2.3 Assay for histone deacetylase activity

The assay for enzymatic deacetylation of histones follows the method proposed by Inoue and Fujimoto (1970). One millilitre aliquots of the fractions to be tested were added to 1 ml portions of ^3H -acetyl histone solution (1 mg/ml containing 3 350 cpm) (4.6.1) and incubated at 37°C for 1 h. The reaction was stopped by the addition of 0.3 ml of 0.1 M HCl containing 0.05 M acetic acid (unlabelled) as a carrier. The released ^3H -acetate was extracted into 3 ml of ethyl acetate and 1.5 ml of the organic layer taken for ^3H -determination.

The fraction with maximum deacetylating activity (fraction 24) was adjusted to pH 8.0 with 0.01 N NaOH since pH 8 is the pH optimum of histone deacetylase (Vidali et al. (1972) and 70 μl (150 μg protein) was incubated with either 20 μg of ^3H -H4 or 70 μg of ^3H -H3/2B/2A prepared as described (4.5.6.2). At the end of 60 min at 37°C , either 50 μl of H_2O -saturated ethyl acetate were added and 25 μl of the organic layer counted or else the incubation tube contents were freeze-dried and aliquots applied to 10 cm polyacrylamide gels and electrophoresed (4.2.5). Radioactivity in the polyacrylamide gels was determined as described in 4.2.6.

4.7

PREPARATION OF ^3H -H4 HISTONE PEPTIDES

4.7.1

Chymotryptic hydrolysis of ^3H histone-H4

Chymotryptic hydrolysis was performed as described by DeLange et al. (1969). About 20 mg of H4 histone (unlabelled) and 1.7 mg (6.4×10^6 dpm) of ^3H -histone H4 (prepared as described in 4.5.6.2.1) were dissolved in 6 ml of water at 40°C and the pH adjusted to 8.0 with NaOH. After chymotrypsin (0.2 mg) was added the pH was maintained at 8.0 by the addition of 0.01 N NaOH with the pH-stat while hydrolysis proceeded for 25 min when the rate of alkali uptake had decreased to a very low level. Since DeLange et al. (1969) found no further base uptake upon the addition of more chymotrypsin (a result confirmed in preliminary experiments), the reaction was terminated by adjusting the pH to 3.0 with glacial acetic acid and the reaction mixture freeze-dried.

4.7.2

Purification of chymotryptic peptides

The purification was based on the method of DeLange et al. (1969), except that Sephadex G-75 was routinely used instead of Sephadex G-25 and G-50. Nomenclature of H4 histone peptides is given in Fig. 3.4.

The chymotryptic hydrolysate was dissolved in 0.5 ml 99% formic acid, diluted to 1 ml with distilled water and subjected to gel filtration as follows : A 1.5 x 150 cm G-75 Sephadex column, packed and equilibrated with 30% acetic acid and run at 160 cm head, flow rate 24 ml/h, was eluted with 30% acetic acid and 3 ml fractions were collected. Aliquots (10 μl) were removed for counting and fractions were pooled as required and freeze-dried. Fractions containing C-1 peptide required further purification and were re-applied to the same column, eluted, pooled and freeze-dried.

4.7.3

Amino acid analysis of chymotryptic peptide mixtures

1 mg of peptide mixture was hydrolysed at 110°C for 24 h in 5.7 N HCl, dried and applied to a Beckman Model 116 amino acid analyser.

Mrs. M. Morgan and Mr. J. Rodrigues are thanked for performing the hydrolyses and amino acid analyses.

4.7.4 Maleylation of chymotryptic peptides

Maleylation of chymotryptic peptide prepared by methods indicated above (4.7.1 and 4.7.2), tryptic hydrolysis and purification of peptides was performed essentially as described by DeLange et al. (1968, 1969a).

Chymotryptic peptide C-1 (2.1 mg) (Peak II from Sephadex G175 columns) (4.7.2) was dissolved in 1.6 ml water at pH 9.0 and 0°C and 8 mg of maleic anhydride (20-fold excess) was added with stirring over 5 minutes while the solution was maintained at pH 9.0 with the pH-stat. After 40 minutes the maleylated peptide was dialysed against 0.1 N NH_4HCO_3 for 2 hours and then against two changes of water at pH 8.0 for 3 hours.

4.7.5 Tryptic hydrolysis of maleylated peptides

The maleylated peptide was hydrolysed in 5.2 ml of water at pH 8.0 and 40°C with 0.3 mg of trypsin for 25 min. The solution was maintained at pH 8.0 by the addition of 0.01 N NaOH during hydrolysis. The tryptic hydrolysis was stopped by lowering the pH to 3.0 with glacial acetic acid, and the mixture was left at 40°C for 40 h to demaleylate in the presence of a drop of toluene. According to DeLange et al. (1969a) demaleylation should occur to more than 90% under these conditions. After demaleylation the peptides were freeze-dried.

4.7.6 Purification of tryptic peptides

4.7.6.1 Gel filtration on G-25 Sephadex

The freeze-dried peptides (4.7.5) were dissolved in 0.5 ml of 99% formic acid, diluted to 1 ml with water, and applied to a 1.5 x 150 cm column of G-25 Sephadex equilibrated and eluted with 30% acetic acid. A pressure head of 80 cm gave a flow rate of 40 ml/h. 3 ml fractions were collected. Aliquots of 5 μl were taken for radioactivity determinations. Fractions were pooled as required and freeze-dried.

4.7.6.2 Paper chromatography

An aliquot of the single radioactive peak obtained from G-25 gel filtration (4.7.6.1) was subjected to paper chromatography according to the method of DeLange et al. (1968).

Descending chromatography was carried out for 20 h in a glass tank capable of holding a sheet of Whatman No. 3MM paper (46 x 57 cm). Peptide T(M)-2 and T(M)-5 mixture was applied to the paper as a small spot and the paper was placed in the chamber and allowed to equilibrate overnight, before development using pyridine:l-butanol:glacial acetic acid:water (15:10:3:12). Valine (50 µg) was used as a standard. After 20 hours the paper was removed from the chamber, air dried and sprayed with 1% ninhydrin in acetone (Zweig and Sherma, 1972). To develop the spots the paper was warmed to 35°C for 5 min. Radioactivity was determined either by cutting the paper into 1 cm strips and oxidising in the sample oxidiser, or else by elution with 30% acetic acid followed by counting. If samples were to be used for amino acid sequence determination, the area of paper with the peptide to be sequenced was cut out before ninhydrin spraying to preserve the free α -NH₂ group.

4.8 AUTOMATIC EDMAN DEGRADATION

4.8.1 Sequencing of protein and peptides

A Beckman model 890 sequencer was used.

2 mg ³H-histone H3 dimer (4.5.6.2.2) dissolved in water (130 nmole, 3.2 x 10⁶ dpm) was subjected to sequence analysis.

The mixture of ³H-histone H4 peptides T(M)-2 and T(M)-5 (4.7.6) was sequenced together with 2.6 mg of unlabelled histone H4 from calf thymus. 0.09 mg of T(M)-2/T(M)-5 (28 nmole of each peptide, 1.1 x 10⁵ dpm) and unlabelled calf histone H4 were allowed to couple with a 5-fold excess of sulfonated-phenylisothiocyanate at 55°C for 30 min in 1 ml of 3(N,N-dimethylamino) propyne buffer (Braunitzer et al., 1970), before application to the sequencer cup.

The sequential degradation, gas chromatographic identification of PTH-amino acids, hydrolysis of PTH-amino acids with subsequent amino acid analysis (Beckman analyser model 116) was done by Dr. W. Brandt, Mrs. Margaret Morgan and Mr. J. Rodrigues whose help is acknowledged. Protein- or peptide programs were used as described by Brandt and von Holt (1974).

4.8.2 Radioactivity determinations of PTH-amino acids

After conversion to the PTH-amino acids, the ethyl acetate and aqueous layers were separated, dried, the residues resissolved in methanol and aliquots were counted for radioactivity (4.2.4).

4.8.3 Thin layer chromatography of PTH-lysine and PTH-N-ε-acetyllysine

Thin layer chromatography was performed on silica gel F₂₅₄ 20 x 20 x 10 cm sheets (Merck Art 5735/0025 TLC plastic sheets, 0.25 mm layer thickness) using chloroform:methanol:ethanol (90:7:1) as developing solvent.

PTH-amino acids were detected under ultraviolet light. Chromatography was carried out at room temperature in glass chambers, lined with filter paper on 2 sides and equilibrated overnight before use. Multiple development of the plate was used.

The remainder of the ethyl acetate layers (4.8.2) containing PTH-amino acids from the sequencer were dried and redissolved in methanol and spotted onto the plates. Material containing at least 1000 dpm was applied to each spot. Authentic PTH-ε-acetyl lysine prepared as described above (4.1) was co-chromatographed with each sample. To locate radioactive PTH-acetyllysine 1 cm squares were scraped off the plates and the radioactivity of the material removed from the plate was determined (4.2.4).

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